

The 5-HT₄ receptor

-a foetal gene re-expressed in cardiomyocytes from failing hearts?

**A real-time quantitative
reverse transcriptase
polymerase chain reaction study**

**Thesis in pharmacology
for the *cand. pharm.* degree**

by

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ABBREVIATIONS

| Abbreviation | Full name |
|------------------------|---|
| 5-HT | 5-hydroxytryptamine (serotonin) |
| 5-HTT | Serotonin transporter (SERT) |
| A ₂₆₀ | Absorbance at 260nm |
| A ₂₈₀ | Absorbance at 280nm |
| AB | Aorta-banded (hypertrophied non-failing animals) |
| ABHF | Aorta-banded heart failure |
| AC | Adenylyl cyclase |
| ACE | Angiotensin converting enzyme |
| ADH | Anti diuretic hormone |
| ANP | Atrial natriuretic peptide |
| AR | Adrenergic receptor |
| Arbp0 | Acidic ribosomal phosphoprotein P0 |
| AT | Angiotensin |
| ATP | Adenosine triphosphate |
| BNP | Brain natriuretic peptide |
| BSA | Bovine serum albumin |
| BW | Body weight |
| C | Cytidine |
| cAMP | Cyclic adenosine monophosphate |
| cDNA | Complementary deoxyribonucleic acid |
| CHF | Congestive heart failure |
| CHFR | Centre for Heart Failure Research |
| CM | Cardiomyocytes |
| CNS | Central nervous system |
| C _t | Threshold cycle |
| C-terminal | Carboxy terminal |
| DAG | 1,2-diacylglycerol |
| dATP | Deoxy adenosine triphosphate |
| dCTP | Deoxy cytidine triphosphate |
| D-Glucose | Deoxy glucose |
| dGTP | Deoxy guanine triphosphate |
| DNA | Deoxy ribonucleic acid |
| DNase | Deoxy ribonuclease |
| dNTP | Deoxy nucleotide triphosphate |
| dT | Deoxy thymidine |
| DTT | Dithiothreitol, Cleland's reagent |
| dTTP | Deoxy thymidine triphosphate |
| FRET | Förster (or Fluorescence) resonance energy transfer |
| G | Guanidine |
| GAPDH | Glyceraldehyde-3-phosphate-dehydrogenase |
| GI | Gastrointestinal |
| G _i protein | Inhibitory G protein |
| GPCR | G protein-coupled receptors |
| G protein | Guanine nucleotide binding protein |
| G _s protein | Stimulatory G protein |

| | |
|------------------|--|
| IP ₃ | Inositol triphosphate |
| LV | Left ventricular |
| LVEDP | Left ventricular end-diastolic pressure |
| LVSP | Left ventricular systolic pressure |
| LVW | Left ventricular weight |
| M | Internal control gene-stability measure |
| MHC | Myosin heavy chain |
| mRNA | Messenger ribonucleic acid |
| NCM | Non-cardiomyocytes |
| NE | Norepinephrine |
| N-terminal | Amino terminal |
| NYHA | New York Heart Association |
| PCR | Polymerase chain reaction |
| PIP ₂ | Phosphatidylinositol-4,5-bisphosphate |
| PKA | Protein kinase A |
| Polr2A | Polymerase (RNA) II (DNA directed) polypeptide A |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| Rpl4 | Ribosomal protein L4 |
| Rpl32 | Ribosomal protein L32 |
| rRNA | Ribosomal ribonucleic acid |
| RT | Reverse transcriptase |
| RT-PCR | Real-time polymerase chain reaction |
| SEM | Standard error of the mean |
| SERCA2 | Sarco (endo) plasmic reticulum Ca ²⁺ ATPase 2 |
| SERT | Serotonin transporter (5-HTT) |
| SSIII | Super Script III |
| SV | Stroke volume |
| TAE | Tris-acetate EDTA |
| Tbp | TATA box binding protein |
| UV-light | Ultra violet light |

1. SUMMARY

Myocardial remodelling and failure are associated with alterations in gene expression characterised by the re-expression of a foetal gene pattern. This often involves a shift to foetal isoforms of contractile proteins such as e.g. MHC (myosin heavy chain). Little is known about whether or not serotonin receptors are part of this foetal gene program or about their expression in cardiomyocytes in heart failure.

The aim of this study was to evaluate the expression level of 5-HT_{4(b)}, 5-HT_{2A} and 5-HT_{2B} receptors and the serotonin transporter (SERT, 5-HTT) in foetal, neonatal and adult rat hearts as well as in failing and Sham operated rat hearts (Sham).

To determine the localisation of the 5-HT receptors regulated in hypertrophic heart failure, 5-HT_{4(b)}, 5-HT_{2A} and 5-HT_{2B} expression in cardiomyocytes (CM) and non-cardiomyocytes (NCM) from Sham and aorta-banded animals were also studied.

Foetal and neonatal rat hearts were taken out at day 3 and 1 ahead of expected birth and 1, 3 and 5 days after birth, respectively. Hearts were also taken from adult rats at the age of 113 days. Systolic heart failure was induced in Wistar rats by coronary artery ligation. Sham operated rats were run in parallel. Real-time quantitative reverse transcriptase (RT) polymerase chain reaction (PCR) was run using dual labelled probes. The mRNA expression level of 5-HT_{4(b)}, 5-HT_{2A}, 5-HT_{2B} and SERT was determined and normalised to the expression of six normalisation genes by the use of geNorm software. The results were compared to adult hearts or Sham.

The CM and NCM fractions were isolated from Wistar rat hearts exposed to banding of the ascending aorta. By real-time quantitative RT-PCR the expression level of the 5-HT_{4(b)}, 5-HT_{2A} and 5-HT_{2B} receptor as well as β_1 - and β_2 -adrenergic receptor (AR) of CM and NCM were analysed. The results were normalised to GAPDH (Glyceraldehyde-3-phosphate-dehydrogenase) and compared to Sham.

For the 5-HT_{4(b)} receptor, the expression level in failing rat heart was modest compared to the foetal heart. However, it was comparable to the neonatal genotype, proposing 5-HT_{4(b)} as a

reactivated foetal gene. In the aorta-banded rat heart model, the 5-HT_{4(b)} receptor mRNA expression level was six times higher in both the NCM and CM of ABHF rats, compared to Sham. At time of birth, associated with an acute increase in wall stress, the 5-HT_{2A} and 5-HT_{2B} receptor mRNA expression were increased. For the 5-HT_{2A} receptor the expression level was elevated 2- and 3-fold in the NCM and CM, respectively, both in hypertrophied and failing heart. The 5-HT_{2B} mRNA expression level was four times higher in NCM from heart failure rats but decreased by 50% in the CM heart failure group, compared to Sham. SERT was not regulated in foetal and neonatal hearts, or heart failure.

2. INTRODUCTION

2.1 Heart failure

Congestive heart failure (CHF) is a complex of signs and symptoms that occurs when the heart fails to pump an adequate cardiac output. The heart can no longer pump blood according to the demands of the body. Symptoms such as air hunger (dyspnoea), feeling of tiredness (fatigue) and oedema among others, may occur.

There are several reasons for induction of heart failure; Pumping against a chronically elevated arterial pressure in hypertension (diastolic heart failure) and structural damage and impaired cardiac function due to decreased coronary blood flow (systolic heart failure) are the two most common ones. Most cases of heart failure can be explained by well recognised etiologic factors, though seemingly healthy persons may harbour risk factors for the later development of the disease. Cardiac risk factors include obesity, diabetes, high blood pressure, high cholesterol, smoking and genetics.

A fundamental response to myocardial injury or altered loading conditions includes remodelling of the heart so that the size, shape and function of the affected chambers are distorted. This is accompanied by a constellation of biological changes. Together, these multiple alterations are very important to both the morbidity and mortality of the patients (Francis and Tang, 2003).

Approximately 1-2% of the population is at any time affected by heart failure. It becomes more common with increasing age, and approximately 6% of people over the age of 64 suffer from heart failure or have had a failure episode. For people over the age of 74 the risk of heart failure is 10%. Heart failure patients are frequently in contact with the hospital and heart failure is a serious medical problem, both for the patient it self and the public health service (Gullestad and Westheim, 2004).

Heart failure patients are often classified according to the New York Heart Association (NYHA) functional classification system which relates symptoms to everyday activities and quality of life (Table 1).

Table 1: The NYHA classification of congestive heart failure

| Class | Patient symptoms |
|----------------------|---|
| Class I (Mild) | No limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitations or dyspnoea. |
| Class II (Mild) | Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in fatigue, palpitations or dyspnoea. |
| Class III (Moderate) | Marked limitation of physical activity, Comfortable at rest, but less than ordinary activity causes fatigue, palpitations or dyspnoea. |
| Class IV (Severe) | Unable to carry out any physical activity without discomfort. Symptoms of cardiac insufficiency at rest. If any physical activity is undertaken, discomfort is increased. |

2.1.1 Systolic and diastolic heart failure

Heart failure may be caused both by systolic and diastolic dysfunction. Often, both kinds will be present at the same time. In diastolic heart failure there are problems with ventricular filling, whereas in systolic failure there are problems with ventricular ejection.

It is estimated that 20-50% of patients with heart failure have preserved systolic function or a normal left ventricular ejection fraction. Although such hearts contract normally, relaxation (diastole) will be abnormal. Compared to systolic dysfunction, diastolic heart failure has been studied in relatively few clinical trials. However, the mortality among patients with diastolic heart failure may be as high as the mortality among the systolic heart failure patients. The rates of hospitalisation in the two groups are equal (Jessup and Brozena, 2003).

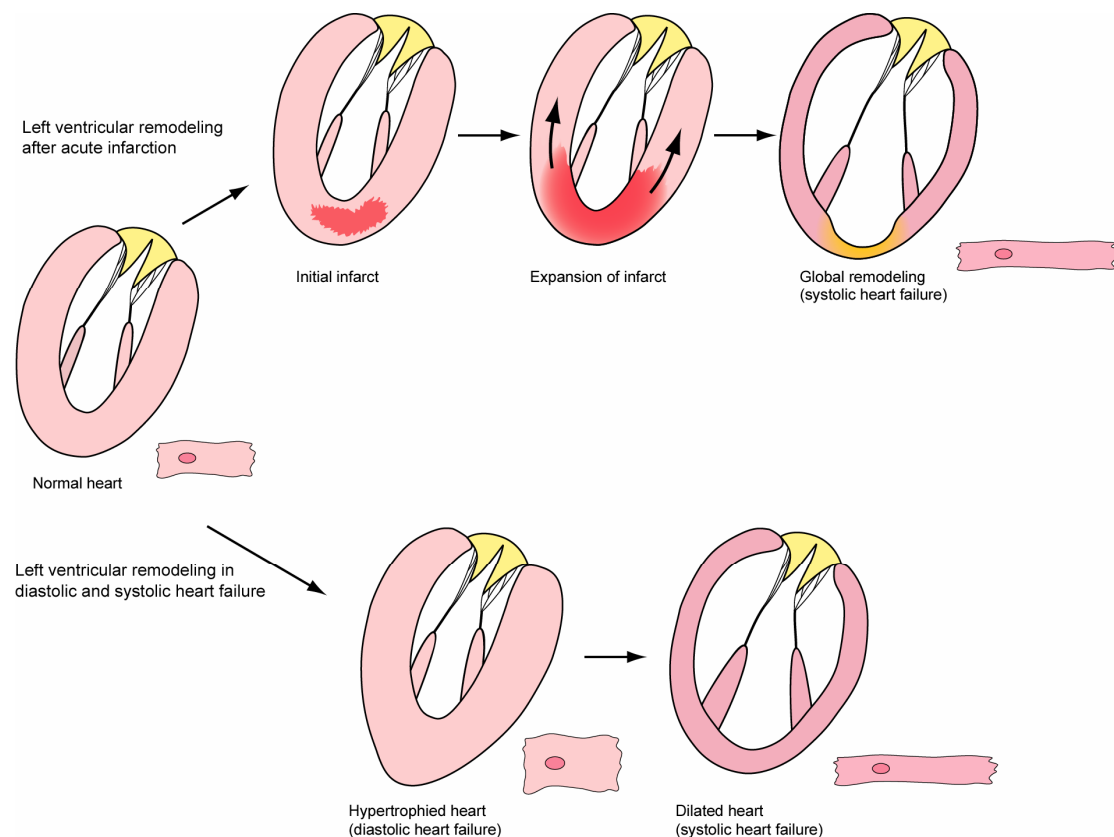


Figure 1: Progression of ventricular remodelling after myocardial infarction (upper) or hypertrophic (lower) onset of the index event. *Upper panel:* An acute infarction leads to local necrosis and peripheral ischemia to site of infarction. Upon expansion of the affected myocardium the loss of contractile tissue is substantial and the ventricle wall tissue becomes thinner due to impaired cardiac function and increased end-diastolic pressure. A sustained exposure to these conditions causes dilatation and global remodelling of the ventricle. *Lower panel:* Hypertrophy normally develops over time with an increase in ventricular wall but almost normal sized ventricular cavity to compensate e.g. increased pumping resistance. The compensatory concentric hypertrophy displaces the tissue composition (contractile vs. non-contractile tissue) which leads to ventricular dilatation. From Brattelid (unpublished) with permission.

Systolic heart failure

Systolic heart failure tends to affect men more often than women and they are typically between 50 and 70 years old. Myocardial damage induced by e.g. myocardial infarction is the main source leading to systolic dysfunction. After a myocardial infarction, the acute loss of myocardial cells results in abnormal loading conditions that involve not only the border zone of the infarction, but also remote myocardium. The remodelling continues for months after the initial insult, and the eventual change in the shape of the ventricle becomes deleterious to the overall function of the heart as a pump. The pathophysiological changes are characterised by a decrease in cardiac contractility, a lower stroke volume at any given end-diastolic volume. These abnormal loading conditions induce dilatation and change the shape of the ventricle,

rendering it more spherical, as well as causing hypertrophy with both a widening and lengthening of the individual myocytes.

In dilated cardiomyopathy, the remodelling process with ventricular dilatation and hypertrophy occurs without the initial apparent myocardial injury observed after a myocardial infarction (Jessup and Brozena, 2003). The myocytes lengthen without widening, making a bigger chamber volume versus wall thickness.

Diastolic heart failure

Diastolic heart failure is largely a product of the aging population. The typical diastolic heart failure patient is often female, obese and frequently has diabetes (Jessup and Brozena, 2003). Diastolic heart failure occurs in patients with long standing hypertension and left ventricular hypertrophy. However, it may also occur in patients with hypertrophic and restrictive cardiomyopathy, and some of these patients may be quite young (Francis and Tang, 2003).

In diastolic dysfunction the wall of the left ventricle has reduced compliance. It is abnormally stiff and has a reduced ability to fill adequately at normal diastolic filling pressure. The result is a reduced stroke volume. As mentioned earlier, one cause of diastolic heart failure is hypertension, and it is the chronic pumping against this elevated pressure that makes the ventricle stiff and less able to expand.

The diagnosis of diastolic heart failure requires three conditions; 1) Presence of signs and symptoms of heart failure, 2) Presence of normal or slightly reduced left ventricle ejection fraction ($>50\%$) and 3) Presence of increased diastolic pressure or impaired filling caused by delayed isovolumic relaxation or elevated stiffness (Mandinov et al., 2000).

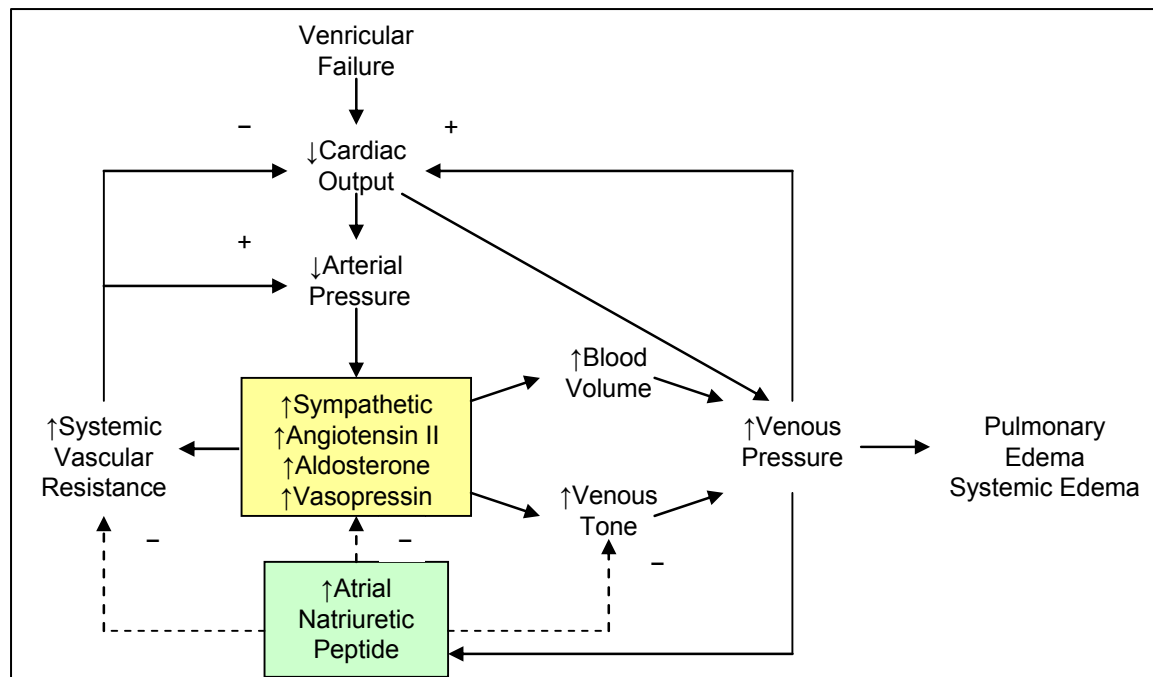
2.1.2 Pathophysiology of heart failure

Heart failure may be caused by factors originating from the heart (intrinsic disease or pathology) or from external factors that place excessive demands upon the heart. Acute heart failure is characterised by a rapid onset of failure that can generally be reversed by therapeutic interventions. Acute failure may for example result from acute myocardial infarction. Chronic heart failure, on the other hand, results from the heart undergoing adaptive responses to a precipitating cause. It is this cardiac response that ultimately may lead to impaired function.

The failing heart tries to compensate for the lowered cardiac output, but can only cope with this situation for a short period of time. There will also be retention of water and salt and a higher peripheral resistance. However, in a prolonged perspective, these compensatory mechanisms actually contribute to the progression of the disease and increase the cardiac work load (Gullestad and Westheim, 2004). The cardiovascular system maintains arterial pressure and perfusion of vital organs (heart, brain and kidney) by different compensatory mechanisms. The most important ones are:

- **The Frank-Starling Mechanism.** Increased venous return increases the ventricular filling (end-diastolic volume) and therefore preload which is the initial stretching of the cardiac myocytes prior to contraction. Myocyte stretching increases the sarcomere length which causes an increase in force generation. This mechanism enables the heart to eject the additional venous return, thereby increasing stroke volume (SV).
- **Myocardial hypertrophy.** The mass of contractile tissue is increased, helping SV to increase.
- **An activation of the neurohumoral system.** This includes activation of sympathetic nerves and the renin-angiotensin system. An increased release of vasopressin (antidiuretic hormone, ADH) and natriuretic peptides (atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP)) also occurs.

Renin is released from the kidney in response to reduced renal perfusion, and circulating renin leads to the formation of angiotensin (AT) 1 and 2, a vasoconstrictor which in turn prompts adrenal aldosterone release. Aldosterone retains salt and water in the kidney and so expands blood volume and thereby increases preload. Vasopressin is also released and contributes to preserve the cardiac function by increasing vasoconstriction and water retention. It is the expanded blood volume that promotes the release of a natural vasodilator, ANP from atrial myocytes to counteract the increased preload by way of attenuation. With time, all of these compensatory mechanisms will increase both the ventricular afterload and preload to a point where stroke volume is depressed and oedemas occur. The walls will also enlarge to much making the blood-flow very difficult. To summarise, the pathophysiology of heart failure involves changes in cardiac function, neurohumoral status, systemic vascular function and blood volume (Figure 2).



2.1.3 Medical treatment

Heart failure is a very serious condition, and medical treatment can be very complicated. Some patients with heart failure will die within a short period of time, even though the medical treatment has been fully correct. A patient's functionality, using modern heart failure treatment, will normally improve one level in the NYHA-system. The survival is also increased, but is limited in severe heart failure of level III or IV (Gullestad and Westheim, 2004).

With some exceptions, all heart failure conditions should be treated medically, but non-medical treatment such as salt and water restrictions and to stop smoking is also important to improve heart function. It is uncertain if there are any advantages for patients in NYHA-class I to use medical heart failure treatment if their ejection fraction is normal (Gullestad and Westheim, 2004).

Increased understanding of CHF pathophysiology the last decades have caused dramatical changes in pharmacotherapy; from a hemodynamic model with treatment aimed at correcting hemodynamic defects, to a neurohumoral (often called neurohormonal) model with treatment aimed at preventing maladaptive, biological changes (Braunwald and Bristow, 2000). Altered

neurohormonal activity (norepinephrine (NE), renin, angiotensin and aldosterone) is an important characteristic of heart failure, and increased activity of the sympathetic and renin-angiotensin systems are considered as important factors in the development of the disease and powerful predictors of shortened survival (Gullestad and Westheim, 2004). Since the 1980s, agents that counteract these systems, such as ACE inhibitors and β -AR-blockers, have been used, but still the mortality and morbidity is high for heart failure patients (Gullestad and Madsen, 2004). The current available neurohormonal antagonists may not antagonise all of the biologically active systems that become activated during heart failure, and antagonising alternative neurohumoral systems might be beneficial in addition to current treatment regimens (Qvigstad, 2004). Lately, the remodelling of the myocardium, the molecular-biological model, has been the one given the most attention (Gullestad and Madsen, 2004).

The principle of treatment is to use medicals that have documented effect on symptoms and prognosis, and at the same time to use the simplest regimen. Today, almost every heart failure patient is treated with at least an angiotensin converting enzyme (ACE) inhibitor and a β_1 -AR blocker. In addition they are often given diuretics and digitalis among others. The combinations depend on whether the patient has had other diseases that will affect the treatment and underlying causes or type of CHF.

Before developing drugs, it is necessary to fully understand the pathophysiology of heart failure as well as the underlying mechanisms, and increased interest is turned to find the genetic factors involved in heart failure. Several studies have focused on polymorphisms and identification of gene defects that directly or indirectly are involved in the pathophysiology of the condition, and which may explain the inheritable part of CHF. An earlier recognition and correction of risk factors would provide for a more robust and favourable outcome (Francis and Tang, 2003).

2.2 G protein-coupled receptors

2.2.1 Structure and classification of G protein-coupled receptors

G protein-coupled receptors (GPCRs) form the largest family of cell-surface receptors and mediate responses to a variety of ligands and stimuli, e.g. biogenic amines, nucleosides, lipids, amino acids and peptides, light and ions. By interacting with G proteins, GPCRs

mediate slow neurotransmission and affect metabolic processes by controlling the activity of enzymes, ion channels and vesicle transport. Approximately 50% of all active drugs are ligands for GPCRs. GPCRs mediate crucial cardiac effects both in the normal and failing heart, and altered levels of GPCRs involved in regulation of heart function may explain some of the differences seen in failing versus non-failing hearts.

GPCRs share a common overall structure, despite the diversity of the signal molecules that bind to them. The structure consists of seven transmembrane α -helices, three extracellular loops (exoloops), three intracellular loops (cytoloops), an extracellular amino (N-) terminal and an intracellular carboxy (C-) terminal. Two cysteine residues in the extracellular loops are conserved in most GPCRs and form a disulfide link that is probably important for packing and stabilisation of the protein conformation (Bockaert and Pin, 1999).

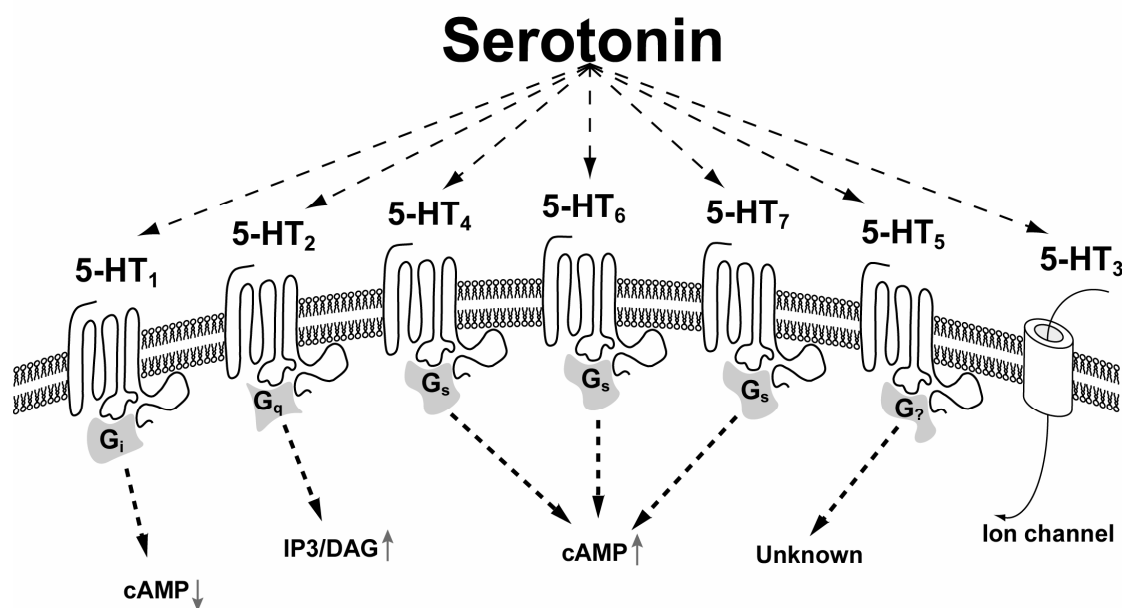


Figure 3: The seven classes of serotonin receptors. The serotonin receptors are divided into seven classes based on their sequence, pharmacological profile and signal transduction mechanism. From Brattelid (unpublished) with permission.

2.2.2 Serotonin receptors

Serotonin mediates intercellular signalling in the central nervous system (CNS) and the periphery through a diverse group of receptors named 5-HT₁ through 5-HT₇. The receptors are classified according to their sequence, pharmacological profile and signal transduction mechanisms. With exception of the 5-HT₃ receptor, they all belong to the GPCR family. The

5-HT₂ family stimulates hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) via G_q, while the 5-HT₄, 5-HT₆ and 5-HT₇ receptors activate adenylyl cyclase (AC) through activation of G_s-protein (stimulatory G protein) (Figure 4) similar to β -adrenergic receptors.

Many of the serotonin receptors are encoded by a single exon, whereas others undergo alternative mRNA splicing of exons giving rise to receptor splice variants. Several splice variants have been described since the rat 5-HT_{4S} (“short”) and the 5-HT_{4L} (“long”) receptors were cloned (Gerald et al., 1995) and later renamed 5-HT_{4(a)} and 5-HT_{4(b)}, respectively. Hitherto, eight human C-terminal splice variants of the 5-HT₄ receptor have been identified and cloned; 5-HT_{4(a)} (Claeysen et al., 1997) (Blondel et al., 1997), 5-HT_{4(b)} (Van den Wyngaert et al., 1997) (Blondel et al., 1998) (Bach et al., 2001), 5-HT_{4(c)} and 5-HT_{4(d)} (Blondel et al., 1998), 5-HT_{4(e)} and 5-HT_{4(f)} (Bender et al., 2000), 5-HT_{4(g)} (Bender et al., 2000), originally called 5-HT_{4(e)} (Claeysen et al., 1999) (Mialet et al., 2000) and 5-HT_{4(n)} (Vilaro et al., 2002). In addition, Bender et al. (2000) found an exon h which could be inserted in the second intracellular loop. Exon h was only observed in combination with the 5-HT_{4(b)} tail and the corresponding receptor was named 5-HT_{4(hb)}. Recently Brattelid et al. (2004a) reported cloning, tissue distribution and pharmacological properties of a novel 5-HT₄ receptor splice variant named 5-HT_{4(i)}. Brattelid et al. (2004a) also reported the existence of two splice variants encoding the human 5-HT_{4(g)} receptor, 5-HT_{4(g1)} and 5-HT_{4(g2)}. All 5-HT₄ receptor splice variants cloned to date show similar pharmacological profiles.

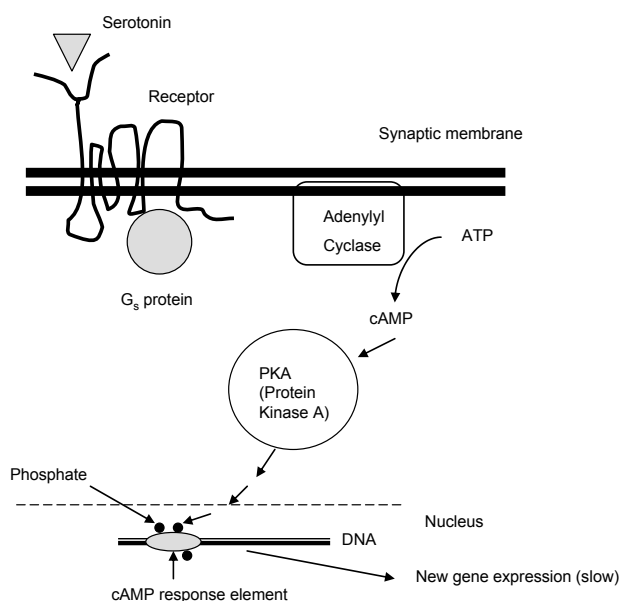


Figure 4: Intracellular signalling mechanisms initiated by activation of the G_s protein-coupled to the 5-HT₄ receptor (in nerve cells)

2.3 Serotonin and serotonin receptors in heart failure

Serotonin is an endogenously occurring vasoactive monoamine which is predominantly produced in enterochromaffine cells of the gastrointestinal (GI) tract and released into the blood (Frishman and Grewall, 2000). Serotonin is removed from blood extracellular space through a high affinity transporter and is mainly stored in platelets. Circulating serotonin can also be taken up by sympathetic neurons and co-released with other biogenic amines (Kaumann, 2000) and can activate 5-HT receptors.

Information about the serotonin system in CHF is limited. However, serotonin can mediate cardioexcitatory effects indirectly through CNS by increasing the release of norepinephrine from nerve terminals, and serotonin elicits positive inotropic, chronotropic and lusitropic effects on the heart. The receptor types that mediate these effects differ greatly between different species. In the rat atrium cardioexcitatory effects of serotonin are mediated via 5-HT_{2A} receptors (Läer et al., 1998) while in porcine and human atrium the effects are mediated through 5-HT₄ receptors (Kaumann, 1990).

The cardiac effects of serotonin have previously been thought to be restricted to the atria because of lack of ventricular effects. However, recent studies have demonstrated the presence of 5-HT₄ receptor mRNA in ventricles in man (Bach et al., 2001) (Brattelid et al., 2004b). A ventricular inotropic response to serotonin mediated by the 5-HT₄ receptor was first demonstrated in ventricles of porcine and explanted human hearts (Brattelid et al., 2004b). The expression of ventricular 5-HT₄ receptor mRNA is enhanced 4-fold in heart failure and associated, in a patient-dependent manner, with serotonin-stimulated increases in contractility, hastening of relaxation and arrhythmias, mediated through 5-HT₄ receptors (Brattelid et al., 2004b). A 5-HT₄ receptor-mediated inotropic response to serotonin was also observed in failing and infarcted rat hearts. The response was not present in normal ventricular myocardium (Qvigstad et al., 2005a). The 5-HT₄ receptor-mediated inotropic response to serotonin in infarcted and failing hearts is associated with an increase in the 5-HT₄ receptor mRNA level.

The apparently similar inotropic role of the 5-HT₄ receptor in failing human and rat ventricle suggests that CHF in rat is a good model for characterisation of the 5-HT₄ receptor in the failing human heart.

In acute heart failure, a situation associated with increased wall stress, an inotropic response to serotonin is also observed. It is mediated through a cAMP-independent mechanism via G_q-coupled 5-HT_{2A} receptors and a cAMP-dependent mechanism via G_s-coupled 5-HT₄ receptors analogous to what is seen for adrenoceptors (α_1 and β) (Qvigstad et al., 2005b).

Concerning the 5-HT_{2B} receptor, Nebigil et al. (2001) have demonstrated a hypertrophic role of the 5-HT_{2B} receptor in the developing mouse heart. Transgenic mice overexpressing the 5-HT_{2B} receptor display a cardiac hypertrophic phenotype, whereas ablation of the 5-HT_{2B} receptor in mice leads to dilated cardiomyopathy.

The 5-HT transporter (5-HTT, SERT) is present with high density in foetal cardiomyocytes mediating an uptake of 5-HT into cells (Sari and Zhou, 2003). Their findings demonstrate a role of SERT by regulating 5-HT in development of foetal heart cells and its implication in the proliferation.

2.4 The foetal gene program

Myocardial remodelling and failure are associated with alterations in gene expression characterised by the expression of a foetal gene pattern (Maytin and Colucci, 2002). This often involves a shift to foetal isoforms of contractile proteins such as myosin heavy chain (MHC) and alterations in calcium handling proteins to levels that are characteristic of foetal myocardium (decreased SERCA2, increased sodium/calcium exchanger). The natriuretic peptides, ANP and BNP are normally expressed in foetal myocardium. They are also markedly induced in remodelling myocardium and are both detectable in the blood where they may be used as an indirect marker for myocardial overload (Maytin and Colucci, 2002). The profile of gene expression in the foetal and failing human heart, however, is largely unknown.

In a study by Razeghi et al. (2001) where they focused on the genes of energy substrate metabolism, they found that the failing adult heart reverts back to a foetal metabolic gene profile by down-regulation of adult gene transcripts rather than by up-regulating foetal genes. The reactivation of the foetal gene program could reflect an adaptive response towards an energy sparing metabolic profile.

Little is known about whether or not 5-HT receptors such as the 5-HT₄ receptor increased in heart failure, could be a part of a foetal gene program reactivated in heart failure.

2.5 Purpose and aims

The significant increase in ventricular 5-HT₄ receptor mRNA and 5-HT₄-mediated inotropic response in CHF may play an important role in the cardiac pathophysiology of heart failure (Brattelid et al., 2004b) (Qvigstad et al., 2005a). A hallmark of heart failure is the transition in gene expression towards a foetal genotype which correlates with loss of cardiac functions. At present there is no evidence of a role of the 5-HT₄ receptor in the foetal heart.

The increased 5-HT₄ mediated inotropic effect in company with an increased 5-HT₄ mRNA level in failing left ventricle (Qvigstad et al., 2005a) implies a co-localisation of these responses to the cardiomyocytes. Due to lack of antibodies with high specificity and sensitivity, localisation of the 5-HT₄ receptor in the failing heart has not been possible.

The aim of this study was therefore to determine if the 5-HT₄ receptor may be a part of the foetal gene program, and whether or not 5-HT₄ receptor mRNA is found in the cardiomyocyte and/or non-cardiomyocyte fractions of failing hearts.

Recent studies in our lab have demonstrated that 5-HT_{2A} is involved in acute CHF and longstanding hypertrophic heart failure (Qvigstad et al., 2005b) (Brattelid et al., 2005a). Nebigil et al. (2003) have demonstrated that the 5-HT_{2B} receptor plays a significant role in heart development and hypertrophy. It was therefore important to also screen whether the 5-HT_{2A} and the 5-HT_{2B} receptors are involved in the foetal gene program as well as their appearance in cardiomyocytes and the non-cardiomyocytes in hypertrophic heart failure. Due to the apparently central role in foetal heart development, the expression level of the serotonin transporter, SERT was also included in the foetal gene program study.

3. MATERIALS AND METHODS

3.1 The rat models

Foetal and neonatal rats

Female Wistar rats were kept in separate cages and housed with a male Wistar rat for 48 hours to ensure a successful mating. At day 3 and 1 ahead of expected birth, the mother was euthanized under anaesthesia (68% N₂O, 29% O₂ and 2-3% isofluran (Abbot Park, Illinois, USA)), and the foetal rats were taken out of the uterus. All the foetal and neonatal rats in each litter were killed by decapitation and hearts were removed and stored in RNAlater (Ambion).

Normal adult rats

Male Wistar rats 113 days of age (age matched with the 6 weeks MI rats described below) were euthanized under anaesthesia (68% N₂O, 29% O₂ and 2-3% isofluran), hearts were removed and immediately stored in RNAlater.

The myocardial infarction (MI) heart failure model

Animals were cared for according to the Norwegian Animal Welfare Act, and two rats (Møllegaard Breeding and Research Centre, Skensved, Denmark) were kept in each cage and housed in a temperature regulated room on a 12:12-h day/night cycle. The animals were given access to food and water *ad libitum*. An extensive infarction was induced by proximal ligation of the left coronary artery in 320g male Wistar rats under anaesthesia (68% N₂O, 29% O₂ and 2-3% isofluran).

The rats were anaesthetised and ventilated as described above, six days after infarction, and they were characterised for left ventricular end-diastolic pressure (LVEDP) measurements. CHF rats with a LVEDP > 15mmHg and a significantly increased heart and lung weight were included in the study (Sjaastad et al., 2000). The CHF classifications were also confirmed with quantitative RT-PCR to screen for increased expression of ANP mRNA.

Sham operated rats (Sham) were run in parallel. They underwent the same surgical procedure, but the coronary artery was not ligated.

Aortic banding model

Animals were cared for according to the Norwegian Animal Welfare Act, and two animals were kept in each cage and housed in a temperature-regulated room on 12:12-h day/night cycle. The animals were given access to food and water *ad libitum*.

Male Wistar rats (Møllegaard Breeding and Research Center, Skensved, Denmark) weighing 200g were anaesthetised with 68% N₂O, 29% O₂ and 2-3% isofluran and ventilated on a respirator (Zoovent, Triumph Technical Services, Milton Keynes, UK). The ascending aorta was dissected free through a hemithoracotomy on the right side. A stenosis of the *aorta ascendens* was induced by a ligation (3-0 silk) which included both the aorta and a steel wire (0.9 mm). Immediately after the ligation the steel wire was removed. This resulted in a ~0.9 mm aortic diameter. Subsequently the chest was closed and the animals were given analgesic drugs. The Sham operated rats were subjected to the same surgical procedure without banding of the ascending aorta.

After six weeks the rats were again anaesthetised and ventilated on the respirator. Echocardiography was performed and the heart excised into saline, weighed and mounted on a modified Langendorf setup. After removal of the left ventricle (LV) posterior papillary muscle, the right ventricle (RV) and LV weights were measured. The aorta-banded (AB) animals were grouped into failing (ABHF) and non-failing animals (AB). As criteria, the ABHF animals had both lung weight (>2.0g) and left atrial diameter (>5.0mm) increased compared to Sham and AB groups. The banded animals that did not fulfil these criteria were divided into 3 groups (AB1, AB2, AB3) based on the degree of LV hypertrophy (LV weight to body weight (BW) ratio).

M-mode, two-dimensional and Doppler echocardiography was performed with a VIVID 7 echocardiograph (GE Vingmed Ultrasound, Horten, Norway (GE)) using a M12L 12MHz linear array transducer (GE) and analyzed essentially as described by Sjaastad (2000).

3.1.1 Tissue sampling

All the cardiac tissue isolated was collected and stored in RNAlater according to the manufactures recommendations until further processing. RNAlater is an aqueous, non-toxic tissue storage reagent that rapidly permeates tissue to stabilise and protect cellular RNA in

situ in unfrozen specimens. Tissue conserved in RNAlater can be stored at -20°C long-term. At 25°C, RNAlater preserves RNA in tissue for up to one week.

Isolation of ventricles from foetal and neonatal hearts

Foetal and neonatal ventricles were dissected free from atria under magnifying glass. Ventricles from each litter were pooled and stored in an excess of RNAlater.

Isolation of 113-day-old rat hearts

Hearts of 113-day-old rats were dissected into right and left ventricle and septum and stored in RNAlater. Only septum was processed further.

Isolation of MI rat heart tissue

Rats, still under anaesthesia, were euthanized, the heart was removed and viable tissue from the left ventricle (mostly septum) was isolated and immediately submerged in RNAlater for storage until further processing. To avoid sampling errors, tissues from left ventricle were collected from the same areas in Sham and CHF rats.

Isolation of cardiomyocytes from banding rat hearts

Ventricular cardiomyocytes were isolated from hearts pre-treated with heparin (170IE in 0.2ml, Leo, Ballerup, Denmark) before excision and perfusion at 37°C with a preoxygenated solution containing (mM) NaCl 130, Hepes 25, D-Glucose 22, KCl 5.4, MgCl₂ 0.5, NaH₂PO₄ 0.4 and insulin 0.01g/ml, pH 7.4. After 1-2 min, collagenase Type II (201U/ml, Worthington) and 0.1 mM Ca²⁺ was added to the solution, and the heart was perfused until the aortic valve ruptured. The LV free wall and the septum was minced and gently triturated at 37°C in the collagenase solution with 1g/l bovine serum albumin (BSA). The cell suspension was filtered (200µm nylon mesh) and sedimented. The supernatant was kept for non-cardiomyocyte fraction, and the pellet was resuspended in solution I containing 0.1mM Ca²⁺ and 1g/l BSA. After sedimentation, the pellet (59% cardiomyocytes) was frozen on liquid N₂. The supernatant from both sedimentations was centrifuged at 120g, and the pellet (86% non-cardiomyocytes) was frozen on liquid N₂.

3.2 Isolation of RNA

3.2.1 Homogenisation

RNA later conserved tissues (100mg/1.5ml Trizol) were washed in 0.9% NaCl to remove salt residuals and homogenised in 2ml tubes (Sarstedt) with Trizol (Invitrogen) and a ceramic particle to grind the tissue, using a shaking machine (Retsch MM301, Retsch) at 30.0 Hz for 20 minutes. Trizol reagent maintains the integrity of the RNA while disrupting cells and dissolving cell components pre-conserved in RNA later. Following homogenisation insoluble material was removed by centrifugation at 12000 x g for 10 minutes at 2-8°C. The resulting pellet contains high molecular weight DNA, extracellular membranes and polysaccharides. (See protocol description 8.2.1)

3.2.2 Phase separation, RNA precipitation and RNA wash

A major goal of nucleic acid isolation is the removal of proteins. Separation can be accomplished due to differences in chemical properties. The highly charged phosphate backbone makes the nucleic acids rather hydrophilic while proteins are more hydrophobic. Addition of chloroform followed by centrifugation in eppendorf tubes loaded with Phase Lock Gel (Heavy 2ml, Eppendorf), isolates RNA in the colourless upper aqueous phase. The proteins will remain in the lower chloroform phase, whereas genomic DNA is found in the organic and aqueous interphase. The RNA was precipitated by mixing the aqueous phase with isopropanol and collected by centrifugation at 12000 x g. The RNA pellets were washed with ethanol and dissolved in RNase-free water (Ambion). RNaseOut (Invitrogen) (1µl/20µl solution) was added to inhibit RNase activity. The samples were stored at -70°C. (See protocol description 8.2.1)

Concerning all procedures:

To avoid introducing exogenous RNases to the samples, all reagents used were RNase-free, and all the equipment was decontaminated with RNase-OFF (AppliChem) routinely. Gloves were worn all the time and changed frequently.

3.2.3 Determination of RNA purity, concentration and quality

The RNA quality was assessed by spectrophotometry and gel electrophoresis. The concentration of RNA in the samples was determined spectrophotometrically by measuring the absorbance at 260nm (A_{260}). The ratio between the absorbance at 260nm and 280nm

(A₂₈₀) gives an estimate of the RNA purity concerning proteins. (See protocol description 8.2.2)

To verify RNA quality, the samples were fixed in formamide and subjected to agarose gel electrophoresis to separate the 28S and 18S rRNA fragments. RNA is a negatively charged molecule, at least at neutral pH, and in an electrical field it will move towards the positive pole. The migration of RNA is dependent on factors such as size, conformation, agarose concentration, buffer concentration and the voltage used. RNA was stained with ethidium bromide and the RNA integrity assessed by visualisation under ultra violet (UV) light. Two distinct bands, one each for 28S and 18S, should be visible when the samples are of high quality and RNA has not been degraded. (See protocol description 8.2.3)

3.3 DNase treatment procedure

3.3.1 DNase treatment

Total RNA isolated by the Trizol-method is not absolutely free from DNA. To remove DNA residuals all samples were DNase treated. The DNase (RQ1 RNase-free DNase (1U/μl), Promega) digests every strand of DNA present, both single and double stranded in an Mg²⁺ buffer (RQ1 RNase-free DNase 10X Reaction Buffer). By removing the DNA a more accurate gene expression analysis is achieved. To terminate the reaction a DNase Stop solution (RQ1 DNase Stop Solution, Promega) was added. Incubation at 65°C for 10 minutes inactivates the DNase enzyme. (See protocol description 8.2.4)

3.3.2 Precipitation and washing of RNA after DNase treatment

The DNase treated RNA samples were precipitated by addition of ammonium acetate, Glycoblue (glycogen coupled to a blue stain, Ambion) and absolute ethanol. Glycogen acts as a carrier to increase the precipitation efficiency from dilute RNA solutions. The blue stain makes the pellets blue and therefore easier to see. Ammonium acetate also helps precipitating the RNA. The precipitate was washed in ethanol and dissolved in RNase-free water. RNaseOut was added before storage at -70°C. (See protocol description 8.2.5)

3.3.3 The use of NanoDrop for RNA purity- and concentration-determination

The NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) is a full-spectrum (220-750nm) spectrophotometer that measures samples down to 1µl with high accuracy and reproducibility. The small sample drop is positioned on the instrument by surface tension alone.

Diluted (1:10) samples of 2µl were used, and whilst running, they were kept on ice. All samples were run twice and RNase-free water was used as a blank. (See protocol description 8.2.6)

3.3.4 The use of Bioanalyzer for quality verification

The Agilent 2100 Bioanalyzer (Agilent Technologies) is a microfluidic-based platform for the analysis of DNA, RNA, proteins and cells by capillary electrophoresis. It is an alternative to gel electrophoresis techniques; delivering fast, reproducible, high quality digital data. An electropherogram (chromatogram) showing the 28S and 18S peaks, is generated for each RNA sample included in the study, and the system automatically calculates the ratio of the ribosomal bands (28S/18S). A maximum of 12 RNA samples are loaded to a chip preloaded with gel-dye mix, a marker (RNA 6000 Nano Marker, Agilent Technologies) and a ladder (RNA 6000 Ladder, Ambion). The samples were diluted with RNase-free water according to the total RNA qualitative detection area (5-500ng/µl) and kept on ice during the preparation procedure. (See protocol description 8.2.7)

3.3.5 Making a standard curve

To make a standard curve for the PCR, the same amount (5µg) of total RNA from six different samples were pooled together in one tube. Based on spectrophotometric quantification, amounts of 10, 7.5, 5, 2.5 and 0.5µg template were pipetted into separate tubes. First strand cDNA synthesis was carried out as described below.

3.4 First strand cDNA synthesis

Single stranded mRNA is too unstable to serve as a template for PCR, and mRNA is therefore transcribed into the much more stable cDNA (complementary DNA). This is all accomplished

by the use of the reverse transcriptase SuperScript III (SSIII, Invitrogen). 5.0µg (7.5µg for the cardiomyocytes and non-cardiomyocytes) RNA was mixed together with oligo dT primers, a dNTP Mix (same amount of dATP, dGTP, dCTP and dTTP, all Invitrogen) and RNase-free water to a volume of 26µl. Heating to 65°C allows the primers to anneal. SSIII, RNaseOut, First-Strand Buffer (Invitrogen) and DTT (Invitrogen) were added to a total volume of 40µl. To synthesise the cDNA a special temperature-program was used:

1. 25°C for 5 minutes
2. 50°C for 60 minutes
3. 70°C for 15 minutes (inactivation of the reaction)

Reactions without reverse transcriptase (-RT reactions) were run in parallel. This was carried out to control for genomic DNA contamination. In these samples SSIII and RNaseOut was replaced by DNase-free water. (See protocol description 8.2.8)

3.5 Primer and probe design

The design of good primers and probes is very important to get an effective amplification of the target gene and reliable signals that are not due to primer dimers, primer-probe dimers or replication of other genes due to unspecific binding. While designing primers and probes there are several guidelines that should be followed (See protocol description 8.2.9). In the transcription process from genomic DNA to mRNA the introns are removed and the exons coupled together, and if it is possible, the primers should be designed over an exon-exon junction. This is to avoid replication of genomic DNA. Different software can be used to design primers and probes with the correct melting temperature and to check for secondary structures, primer dimers and primer-probe binding. With exceptions of some primers and probes for normalisation, primers and probes used in this study were designed by Primer Express software (Version 2.0.0, Applied Biosystems) (See protocol description 8.2.9). Almost all primers and probes were obtained from Invitrogen and Eurogentec, respectively, but the primers and probes for Rpl4, Rpl32 and Arbp0 were obtained as mixes from Applied Biosystems. (See Table 2 for further information)

Table 2: Oligonucleotide sequences used for quantification of different genes by real-time quantitative RT-PCR

| Receptor/Gene | GI number | Primer/Probe | Sequence | Reporter dye | Quencher dye |
|----------------------|-----------|--------------|---|---------------|---------------|
| 5-HT _{2A} | 207067 | Forward | 5' TTC ACC ACA GCC GCT TCA A '3 | | |
| | | Reverse | 5' ATC CTG TAG TCC AAA GAC TGG GAT T '3 | | |
| | | Probe | 5' ATG GAT ATA CCT ACA GAT ATG GTC CAC ACG GCA AT '3 | FAM | TAMRA |
| 5-HT _{2B} | 924649 | Forward | 5' GAA TCC TTT GAT CTA TAC CCT CTT CAA TAA '3 | | |
| | | Reverse | 5' AAT AGA GTG TAC TAG AAC ACT TTC TAA GC ACTT TTA '3 | | |
| | | Probe | 5' AAG CAT TTG GCA GGT ACA TCA CCT GCA A '3 | FAM | Dark Quencher |
| 5-HT _{4(b)} | 924649 | Forward | 5' CAT GTG CTA AGG TAT ACA GTG GAA TGT '3 | | |
| | | Reverse | 5' GCA GCC ACC AAA GGA GAA GTT '3 | | |
| | | Probe | 5' CTG TGA GGT GAC ACC GAC TCT CCC ATT '3 | FAM | Dark Quencher |
| 5-HTT | 1841937 | Forward | 5' GTC ATC TGC ATC CCT ACC TAT ATC ATT '3 | | |
| | | Reverse | 5' GTG GGT GTT TCA GGA GTG ATA CTT T '3 | | |
| | | Probe | 5' AAT AAT CCG CTC CTT AAG TGT CCC CGG AGT '3 | FAM | Dark Quencher |
| ANP | 55711 | Forward | 5' ATC TGA TGG ATT TCA AGA ACC '3 | | |
| | | Reverse | 5' CTC TGA GAC GGG TTG ACT TC '3 | | |
| | | Probe | 5' CGC TTC ATC GGT CTG CTC GCT CA '3 | Yakima Yellow | Dark Quencher |
| β_1 | 220670 | Forward | 5' GCA AGG ACC CGA GTG GA AA '3 | | |
| | | Reverse | 5' CAA ACC AGA GCT GAA CAC TTA GGT T '3 | | |
| | | Probe | 5' TGG AGT CTA TCA CCT GCC TTC TAC TTC ACA CC '3 | FAM | Dark Quencher |
| β_2 | 6978460 | Forward | 5' CTC ATC CCT AAG GAA GTT TAC ATC CT '3 | | |
| | | Reverse | 5' CTG GAA GGC AAT CCT GAA ATC T '3 | | |
| | | Probe | 5' ACT CTG CCT TCA ATC CTC TTA TCT ACT GTC GGA '3 | FAM | Dark Quencher |
| MHC- α | 56654 | Forward | 5' CTC AAA CTC ATG GCC ACA CTC TT '3 | | |
| | | Reverse | 5' GAG CCT TTC TTC TTG CCT CCT T '3 | | |
| | | Probe | 5' CC ACCT ATG CTT CTG CTG ATA CCG GTG A '3 | FAM | Dark Quencher |
| MHC- β | 56656 | Forward | 5' CCT CCC TCA AGC TCC TAA GTA ATC T '3 | | |
| | | Reverse | 5' AAA GGA TGA GCC TTT CTT TGC TT '3 | | |
| | | Probe | 5' TTT GCC CTT GTC TAC AGG TGC ATC AGC T '3 | FAM | Dark Quencher |

| gene | Normalisation- GI-number | Primer/Probe | Sequence | Reporter dye | Quencher dye |
|--------|-----------------------------|-----------------------------|---|---------------|---------------|
| Arbp0 | 11693175 | Forward Reverse Probe | } Mix from Applied Biosystems; Rn00821065_g1 | FAM | Dark Quencher |
| GAPDH | 10190788 | Forward Reverse Probe | 5' CCA AGG TCA TCC ATG ACA ACT T '3 5' AGG GGC CAT CCA CAG TCT T '3 5' CTC ATG ACC ACA GTC CAT GCC ATC ACT '3 | Yakima Yellow | Dark Quencher |
| Polr2A | 62656466 | Forward Reverse Probe | 5' CGT ATC CGC ATC ATG AAC AGT GA '3 5' TCA TCC ATC TTA TCC ACC ACC TCT T '3 5' CCT CCT CCT GCA TCT TG '3 | FAM | Dark Quencher |
| Rpl4 | 11968085 | Forward Reverse Probe | } Mix from Applied Biosystems; Rn00821091_g1 | FAM | Dark Quencher |
| Rpl32 | 6981481 | Forward Reverse Probe | } Mix from Applied Biosystems; Rn00820748_g1 | FAM | Dark Quencher |
| Tbp | 51948367 | Forward Reverse Probe | 5' CCT CTG AGA GCT CTG GGA TTG TA '3 5' GCC AAG ATT CAC GGT GGA TAC A '3 5' CCA CAG CTC CAA AAT A '3 | FAM | Dark Quencher |

3.6 Polymerase chain reaction (PCR)

3.6.1 Basic principles

PCR is an extremely sensitive method to amplify a selected DNA sequence *in vitro*. The method requires two primers, a polymerase enzyme and a dNTP mix. A temperature program is started and the DNA sample is first heated to separate the two strands. In a second step the temperature is lowered just below the melting point of the primers to allow them to hybridise to their DNA sequence. Finally, the temperature is increased to the optimum of the polymerase, usually 68-72°C, to replicate the DNA. In practice, 20-30 cycles of reaction is required for effective DNA amplification. Each cycle doubles the amount of DNA and takes about 5 minutes in an automated temperature cycler.

The polymerase used is a Taq DNA polymerase. This is a thermostable enzyme isolated from the thermophilic bacterium *Thermus aquaticus*. This enzyme survives prolonged exposure to temperatures as high as 95°C. Because of this it will still be active after the denaturation steps in the PCR procedure. Normal DNA polymerase would have denatured together with the DNA at temperatures this high.

3.6.2 Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was accomplished for all the selected genes including the genes used for normalisation. Using a probe, in situ quantification of reaction product is possible. The TaqMan probe is a short singlestranded oligonucleotide sequence that is complementary to one of the strands of the amplicon. At the 5'-end of the probe there is a fused reporter sending out a fluorescent signal. At the other end there is a quencher. Both the reporter and the quencher are fluorophores. When the probe is intact and in solution or bound to its corresponding DNA sequence, the proximity of the quencher will reduce the fluorescent signal sent out by the reporter. This is all due to FRET (Förster (or Fluorescence) resonance energy transfer) where the reporter transfers its energy to the quencher which releases the energy as light of a higher wavelength. The DNA polymerase used for quantitative RT-PCR displays a 5'-3' nuclease activity. This means that it can hydrolyse an oligonucleotide bound to its target sequence. Based on this the reporter is separated from the quencher during the synthesising process. When the reporter separates from the quencher, as when the polymerase replicates the template and hydrolyses the probe, fluorescent signals will be sent out. FRET

will no longer occur when the reporter and quencher are separated. This is detected by the real-time quantitative RT-PCR machine as an increase in emitted light intensity.

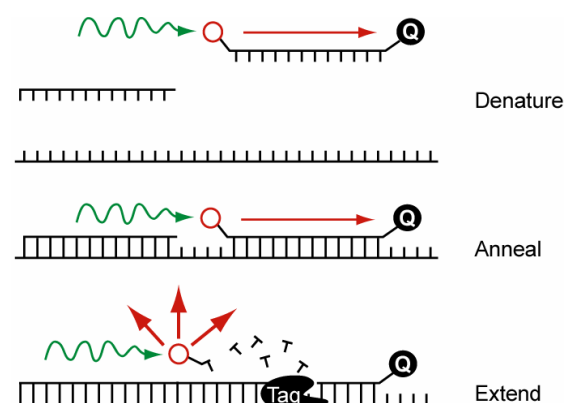


Figure 5: Quantification of mRNA by probe based real-time quantitative RT-PCR. Increasing amount of fluorescence signal is emitted as the sequence is amplified and the reporter and quencher dyes are separated. From Brattelid (unpublished) with permission.

When Taq Man and other dual-labelled probes are used, a 2-step cycling program is run. In the first step the temperature is raised to 94°C to denature the polymerase. In a second step of 60°C, the annealing and extension are all done at once. In addition an initial activation of the polymerase is required. This is accomplished by heating to 95°C for 15 minutes.

The real-time quantitative RT-PCR assays (20µl) were run in a 384-well temperature block operated by an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). (See protocol description 8.2.10)

The Ct (cycle of threshold) value is defined as the cycle number at which the fluorescence is higher than the baseline fluorescence. The higher the starting copy number of the genomic material target, the sooner this is reached.

3.7 Calculations

3.7.1 Analysis of quantitative RT-PCR data

An amplification plot was generated from every sample. This was carried out using Sequence Detector Software (SDS version 2.2, Applied Biosystems). The amplification plots show an increase in fluorescence from the reporter with each PCR cycle. C_t values from each amplification cycle were automatically calculated. The C_t value represents the number of PCR

cycles where fluorescence is first detected above the baseline. The C_t value is obtained in the linear phase of PCR where there are no rate-limiting components. The C_t values were then exported into Excel (Microsoft) for further calculations.

Standard curves for each primer/probe set were made by plotting C_t values versus log initial amount of RNA for the different standard dilutions. This plot gives a straight line (Equation 1) and the slope describes the efficiency of the PCR. If the PCR amplification is optimal, the slope will be -3.3, as 3.3 is the number of cycles that are required to generate a 10 fold increase in product amount.

$$C_t = a (\log Q) + b \quad (1)$$

C_t -cycle of threshold, a is the slope, Q is the initial amount of RNA and b is the intercept on the y-axis.

Equation 1 can easily be rearranged to;

$$\log Q = \frac{C_t - b}{a} \quad (2)$$

Using this equation with the slope a and intercept b calculated from the standard curve and the obtained C_t values for the different samples, the initial amount of target RNA can be calculated.

Normalisation genes

To normalise for differences in the amount of template added, for sample to sample variations in RT-PCR efficiency and for errors in sample quantifications the expression level of each gene analysed are normalised to a so called housekeeping gene. GAPDH (Glyceraldehyde-3-phosphate-dehydrogenase) is probably the most frequently used normalisation gene for quantitative RT-PCR analysis, but actually not very suitable for comparison of CHF vs. Sham hearts (Brattelid et al., 2005b). Due to lack of RNA and knowledge of normalisation genes to use in quantitative RT-PCR, GAPDH only was used for normalisation in the cardiomyocyte and non-cardiomyocyte part of this thesis.

Concerning the foetal gene program study, five normalisation factors additionally to GAPDH were included; Tbp (TATA box binding protein), Polr2A (Polymerase (RNA) II (DNA directed) polypeptide A), Rpl4 (Ribosomal protein L4), Rpl32 (Ribosomal protein L32) and Arbp0 (Acidic ribosomal phosphoprotein P0) (Brattelid et al., 2005b). The normalisation calculations were carried through by the method of Vandesompele et al. (2002) using the Excel based program geNorm. Based on all the normalisation genes, geNorm calculates a normalisation factor for each sample.

Each sample was run in triplicates, and the mean values of the triplicates were used to calculate initial amount of RNA for each sample. To normalise, this value was then multiplied by its corresponding normalisation factor obtained by geNorm.

Finally, values of initial amount of RNA of the target gene relative to the normalisation genes were transferred into GraphPad Prism4 Software (Prism) for statistical comparison and graphical presentation. All data were expressed as mean \pm SEM from n litters or animals. $p < 0.05$ was considered statistically significant (nonparametric Mann-Whitney).

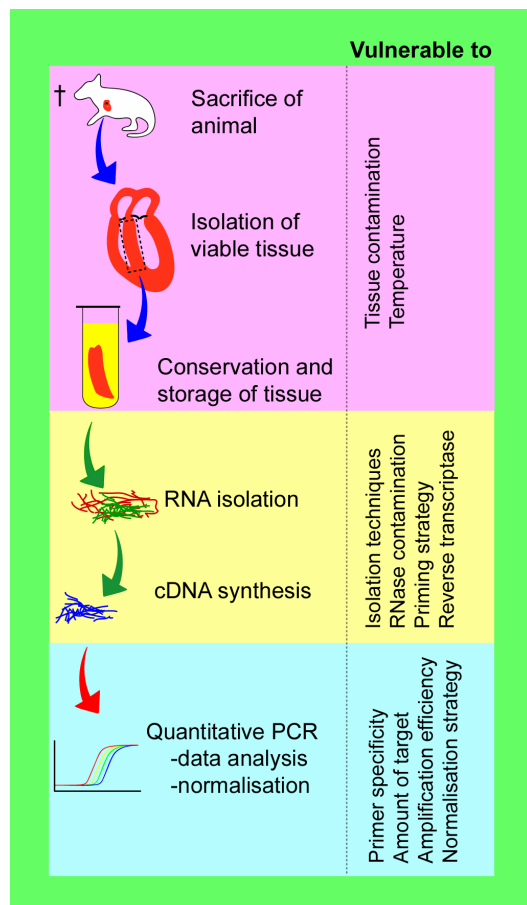


Figure 6: Schematic overview of the methodological steps and problems involved in quantitative RT-PCR. Tissue is sampled immediately after sacrifice of animal and stored until isolation of total RNA. Sampling procedure and temperature exposure is essential to reduce tissue contamination and degradation of the RNA. The method chosen for RNA isolation is usually tissue dependent. Several strategies are available for synthesis of cDNA. Priming strategy, oligo dTs, random hexamers or gene specific primers, and activity and optimal temperature of the RT-enzyme is of importance for the cDNA synthesis and quality of the cDNA. Due to RNase contamination, the RNA is most vulnerable when isolated, but significantly more stable when transcribed into cDNA. The efficiency of the quantitative RT-PCR is dependent on primers and probe specificity and number of copies of gene of interest in sample. Likewise the processing of the results and the normalisation strategy might play an important role for the outcome of quantitative RT-PCR. From Brattelid (unpublished) with permission.

4. RESULTS

To address whether serotonin receptors and the serotonin transporter (SERT, 5-HTT) are involved in the foetal gene program reactivated in heart failure, the level of mRNA encoding 5-HT_{4(b)}, 5-HT_{2A}, 5-HT_{2B} and SERT were analysed by real-time quantitative RT-PCR in foetal, neonatal and adult as well as Sham and CHF (congestive heart failure) operated rat hearts (CHF). To accomplish this extensive study, PCR was run in 384-well plates (384-Well Clear Optical Reaction Plate, Applied Biosystems) on cDNA obtained from the same cDNA synthesis.

To correct for sample variations, all the real-time quantitative RT-PCR data from the genes examined were multiplied by a normalisation factor (based on the expression of six normalisation genes (Table 3)) generated for each sample by the Excel-based program geNorm (Vandesompele et al., 2002). According to Brattelid et al. (2005b) the five housekeeping genes Rpl4, Arbp0, Rpl32, Tbp and Polr2A are all fairly stable normalisation genes in heart. Additionally GAPDH was also used for normalisation. The foetal gene expression levels were compared to the adult (113-day-old) whereas CHF was compared to the Sham group. Both the adult and the Sham group were assigned the value of 1 for simplifying the comparison of groups.

geNorm automatically calculates the internal control gene-stability measure M (defined as the average pairwise variation of a particular gene with all other control genes) for all the control genes included. Genes with the most identical and stable M values have the most stable expression. All normalisation genes should have M values under approximately 1.5 to be stable enough and not to be excluded from incorporation into the calculated normalisation factor value. All the normalisation genes included in the foetal gene program study had M values under this value (Table 3).

Table 3: Achieved M values in geNorm

| GAPDH | Rpl4 | Arbp0 | Rpl32 | Tbp | Polr2A |
|--------------|-------------|--------------|--------------|------------|---------------|
| 0.664 | 0.327 | 0.336 | 0.414 | 0.399 | 0.438 |

4.1 Animal characteristics

4.1.1 The MI heart failure model

The post-infarction heart failure model induced by coronary artery ligation induced hemodynamic changes characteristic for dilated cardiomyopathy with elevated LVEDP and decreased left ventricular systolic pressure (LVSP). These hemodynamic changes resemble those observed previously in this model (Sjaastad et al., 2003). Tachypnoea, pleural effusion and pulmonary congestion were present in the CHF animals. Animal characteristics are summarised in Table 3. All the Sham and CHF rats included in the foetal gene program study were taken from these groups of animals, with $n = 5$ and $n = 6$ for Sham and CHF, respectively.

Table 4: Animal characteristics. Animal characteristics are given as mean values \pm SEM. LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; RV, right ventricle. *CHF vs. Sham $p < 0.05$; ***CHF vs. Sham $p < 0.001$.

| | Sham (n = 9) | CHF (n = 18) |
|---------------------------------|-----------------|--------------------|
| Body weight, g | 380 \pm 10 | 374 \pm 10 |
| Heart weight, g | 1.42 \pm 0.04 | 2.51 \pm 0.10*** |
| Heart weight/ body weight, g/kg | 3.74 \pm 0.14 | 6.71 \pm 0.32*** |
| RV weight, g | 199 \pm 15 | 488 \pm 26*** |
| Lung weight, g | 1.41 \pm 0.04 | 4.33 \pm 0.20*** |
| LVEDP, mmHg | 4.0 \pm 1.3 | 23.0 \pm 1.6*** |
| LVSP, mmHg | 101 \pm 5 | 88 \pm 4* |

4.1.2 Aorta-banded animals

Aortic banding induced a significant increase in left ventricle weight (LVW) in AB 1-3 (hypertrophic group) and ABHF (heart failure group) compared to Sham. Lung weight was increased in ABHF compared to AB 1-3.

The flow velocity also increased to $>6\text{m/s}$ over the aortic stenosis subsequently increasing the LV wall stress, partially compensated by myocardial hypertrophy. Exceeding the compensation limits, the hypertrophic heart will no longer be able to adjust for the increase in afterload, resulting in left ventricular dilatation and CHF.

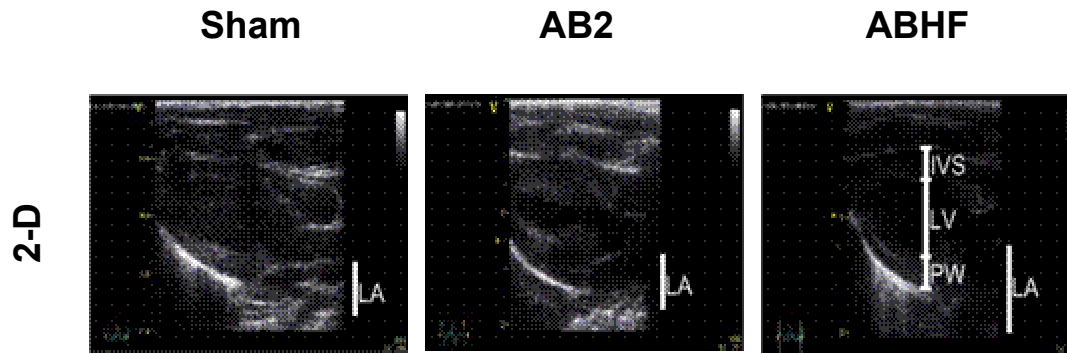


Figure 7: Representative 2-D images of the left ventricle (LV) in end diastole. In AB2 and ABHF the LV wall thickness is increased compared to Sham. In ABHF the LV is dilated compared to AB2. In ABHF the left atrial (LA) diameter is larger than in Sham and AB2. LA -left atrium; IVS -inter ventricular septum; LV -left ventricle lumen; PW -posterior left ventricular wall. Modified after Brattelid et al. (2005a).

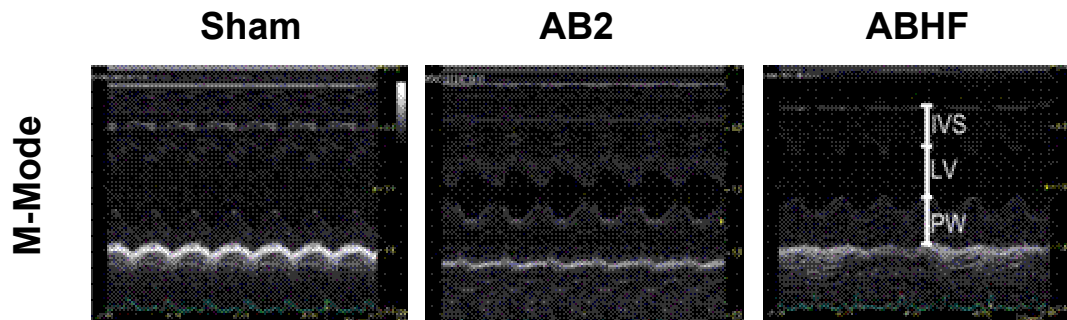


Figure 8: M-Mode images of the LV. The AB2 group develops concentric left ventricle (LV) hypertrophy, and the LV dilates in the ABHF group. IVS -inter ventricular septum; LV -left ventricle lumen; PW -posterior left ventricular wall. Modified after Brattelid et al. (2005a).

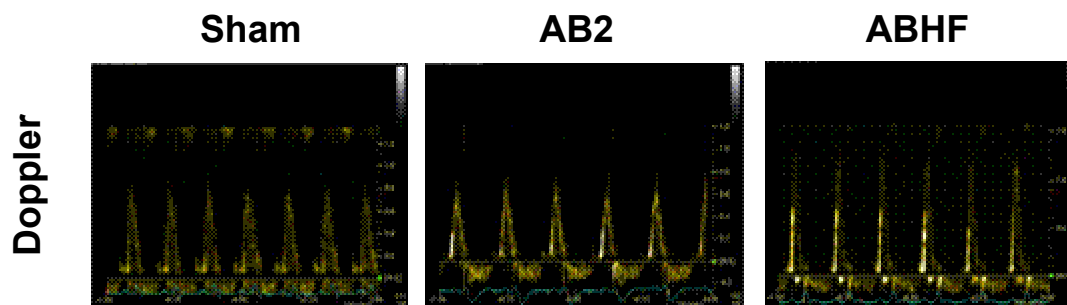


Figure 9: Doppler signals. The deceleration velocity of the mitral valve Doppler signal is increased in ABHF compared to Sham and AB2. Modified after Brattelid et al. (2005a).

4.2 The foetal gene program

4.2.1 The expression of ANP

The level of ANP is known to increase significantly in CHF hearts, and quantification of mRNA encoding ANP was performed to confirm the presence of CHF in the heart failure group.

The level of ANP mRNA was significantly increased in the CHF group when normalised against the six normalisation genes. Compared to adult hearts, ANP mRNA expression was elevated 2-10-fold in foetal and neonatal hearts and about 110-fold in CHF.

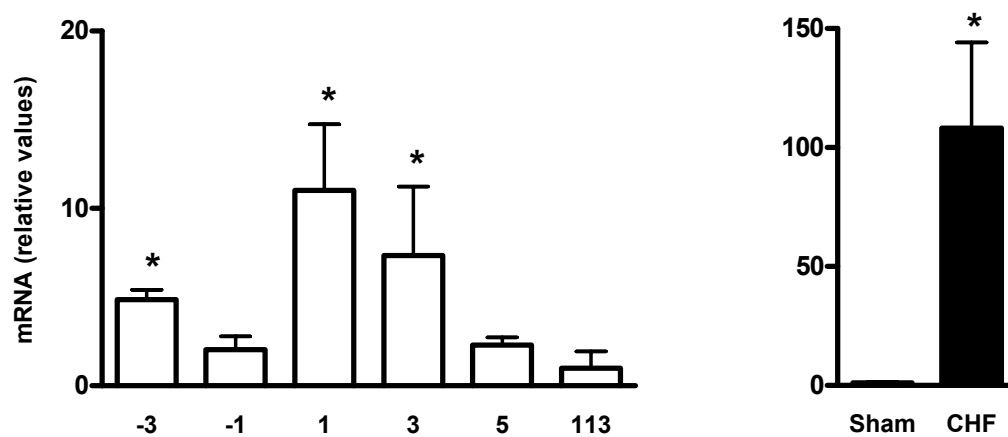


Figure 10: The mRNA expression of ANP in foetal, neonatal, adult and failing rat hearts quantified by the use of real-time quantitative RT-PCR. The foetal and neonatal groups (-3 days to 5 days) are compared to the 113-day-old rat hearts (left panel). Heart failure rats are compared to Sham (right panel). The values obtained are normalised to six normalisation genes (Table 3) by using the method of Vandesompele (2002). The groups of 113-day-old rats and Sham were assigned the value of 1. * $p < 0.05$ vs. 113-day-old rats or Sham

4.2.2 The expression of MHC- α and β

To confirm the foetal, neonatal, adult and CHF cardiac phenotype, MHC- α (α type myosin heavy chain) and MHC- β (β type myosin heavy chain) were analysed.

MHC- α (major isoform in adult heart) mRNA expression was higher in the neonatal and adult hearts compared to the foetal hearts analysed. The significant increase in MHC- α from the foetal expression level was associated with time of birth. MHC- α was decreased in CHF to 25% of the Sham level. This is a level comparable to the foetal ones.

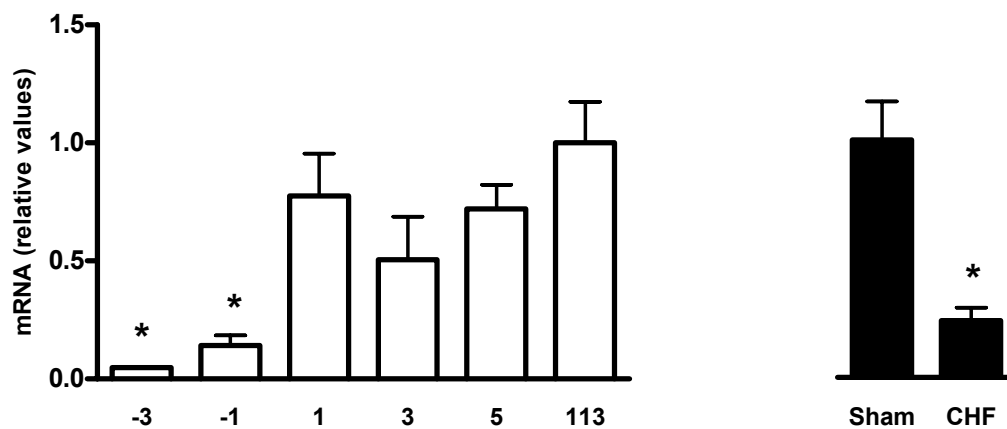


Figure 11: mRNA expression of MHC- α in foetal, neonatal, adult and failing rat hearts quantified by the use of real-time quantitative RT-PCR. The foetal and neonatal groups (-3 days to 5 days) are compared to the 113-day-old rat hearts (left panel). Heart failure rats are compared to Sham (right panel). The values obtained are normalised to six normalisation genes (Table 3) by using the method of Vandesompele (2002). The groups of 113-day-old rats and Sham were assigned the value of 1. * $p < 0.05$ vs. 113-day-old rats or Sham

MHC- β (minor MHC isoform in adult heart) mRNA expression was higher in the foetal and neonatal hearts compared to the adult hearts analysed. A significant increase in MHC- β expression level was associated with time of birth. The MHC- β was significantly increased in CHF by a doubling of the expression level compared to Sham.

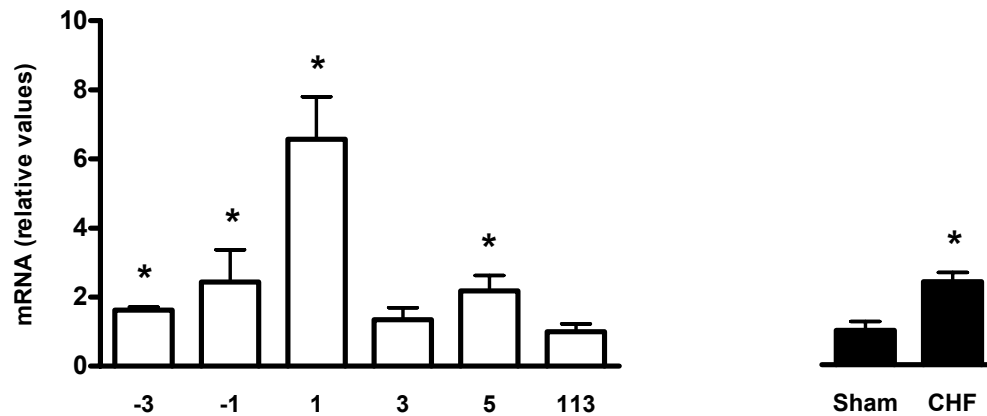


Figure 12: mRNA expression of MHC- β in foetal, neonatal, adult and failing rat hearts quantified by the use of real-time quantitative RT-PCR. The foetal and neonatal groups (-3 days to 5 days) are compared to the 113-day-old rat hearts (left panel). Heart failure rats are compared to Sham (right panel). The values obtained are normalised to six normalisation genes (Table 3) by using the method of Vandesompele (2002). The groups of 113-day-old rats and Sham were assigned the value of 1. * $p < 0.05$ vs. 113-day-old rats or Sham

The expression profiles of ANP, MHC- α and MHC- β mRNA obtained are all in line with previous data indicating reactivation of a foetal gene program in CHF (Razeghi et al., 2001) (Nakao et al., 1997).

4.2.3 Altered expression of serotonin receptor mRNAs in foetal and neonatal development and in CHF

Expression of the 5-HT_{4(b)} receptor mRNA

The 5-HT_{4(b)} mRNA expression level was significantly higher in foetal and neonatal hearts compared to the adult heart. The expression level of 5-HT_{4(b)} mRNA decreased with foetal and neonatal development from day -3 to day 5 and reached a minimum in normal adult hearts (113). In the failing ventricle the 5-HT_{4(b)} receptor expression level was increased 5-fold compared to Sham.

Although the increased expression level of 5-HT_{4(b)} receptor mRNA in CHF was modest compared to that observed in foetal cardiac ventricle, it was comparable to the neonatal expression level.

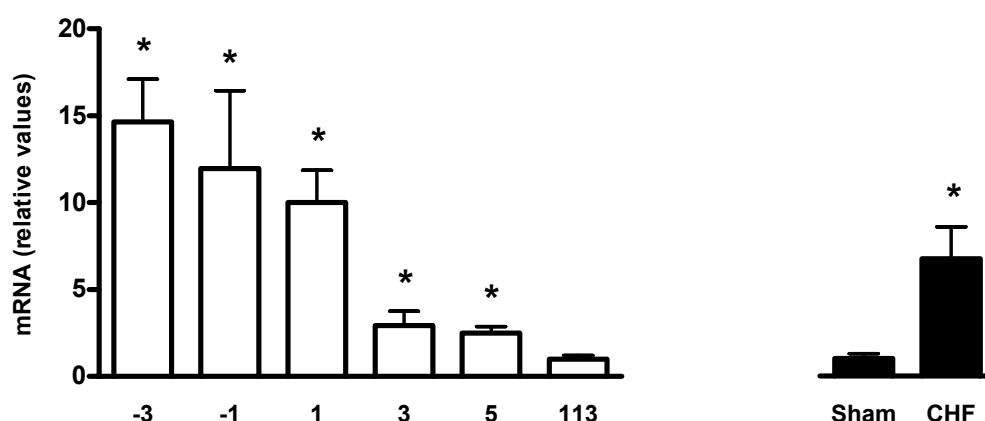


Figure 13: mRNA expression of 5-HT_{4(b)} in foetal, neonatal, adult and failing rat hearts quantified by the use of real-time quantitative RT-PCR. The foetal and neonatal groups (-3 days to 5 days) are compared to the 113-day-old rat hearts (left panel). Heart failure rats are compared to Sham (right panel). The values obtained are normalised to six normalisation genes (Table 3) by using the method of Vandesompele (2002). The groups of 113-day-old rats and Sham were assigned the value of 1. *p < 0.05 vs. 113-day-old rats or Sham

Expression of the 5-HT_{2A} receptor mRNA

The ventricular 5-HT_{2A} mRNA expression level was almost identical in foetal and adult heart. Interestingly, a significant increase of the 5-HT_{2A} receptor mRNA was seen at time of birth, but the expression level declined again during the neonatal period. The time of birth is related to an acute increase in wall stress associated with cardiac growth during the postnatal development. The 5-HT_{2A} mRNA expression level remained unchanged in CHF compared to Sham.

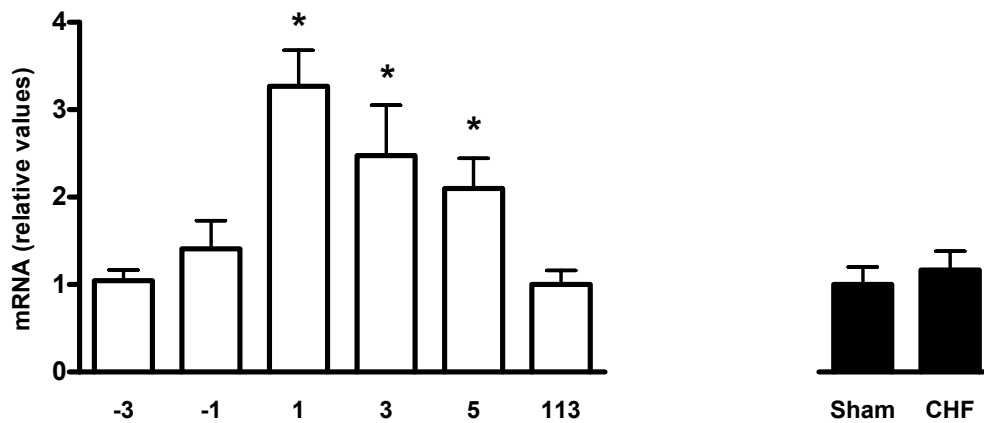


Figure 14: mRNA expression of 5-HT_{2A} in foetal, neonatal, adult and failing rat hearts quantified by the use of real-time quantitative RT-PCR. The foetal and neonatal groups (-3 days to 5 days) are compared to the 113-day-old rat hearts (left panel). Heart failure rats are compared to Sham (right panel). The values obtained are normalised to six normalisation genes (Table 3) by using the method of Vandesompele (2002). The groups of 113-day-old rats and Sham were assigned the value of 1. * $p < 0.05$ vs. 113-day-old rats

Expression of the 5-HT_{2B} receptor mRNA

The ventricular 5-HT_{2B} mRNA expression level was with the exception for time of birth, not changed in foetal and neonatal heart when compared to adult heart. However, at birth (associated with increased wall stress) the mRNA expression level was over twice as high. As observed for foetal an adult heart, there was no change in the 5-HT_{2B} mRNA expression level in CHF compared to Sham.

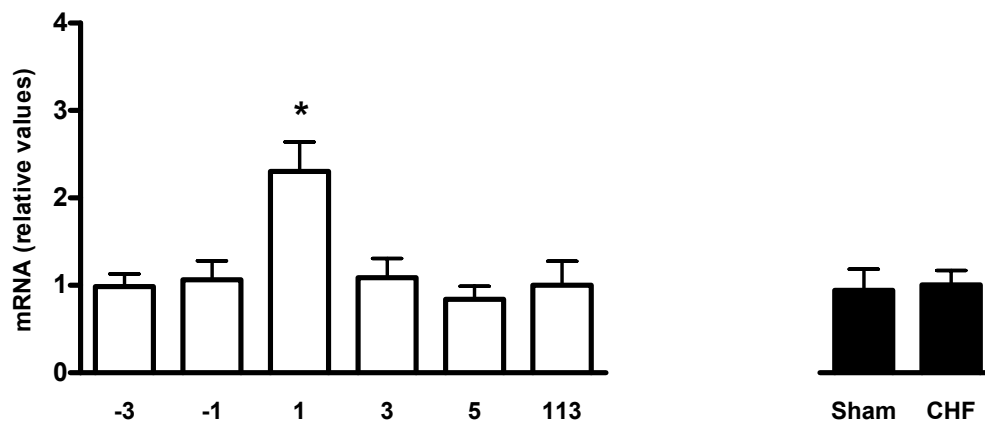


Figure 15: mRNA expression of 5-HT_{2B} in foetal, neonatal, adult and failing rat hearts quantified by the use of real-time quantitative RT-PCR. The foetal and neonatal groups (-3 days to 5 days) are compared to the 113-day-old rat hearts (left panel). Heart failure rats are compared to Sham (right panel). The values obtained are normalised to six normalisation genes (Table 3) by using the method of Vandesompele (2002). The groups of 13-day-old rats and Sham were assigned the value of 1. *p < 0.05 vs. 113-day-old rats

The expression of 5-HTT mRNA

The serotonin transporter mRNA expression level was regulated neither with foetal and neonatal development, nor in heart failure. During development there may seem to be a higher expression of the transporter around birth, and also five days after birth, however, not significant.

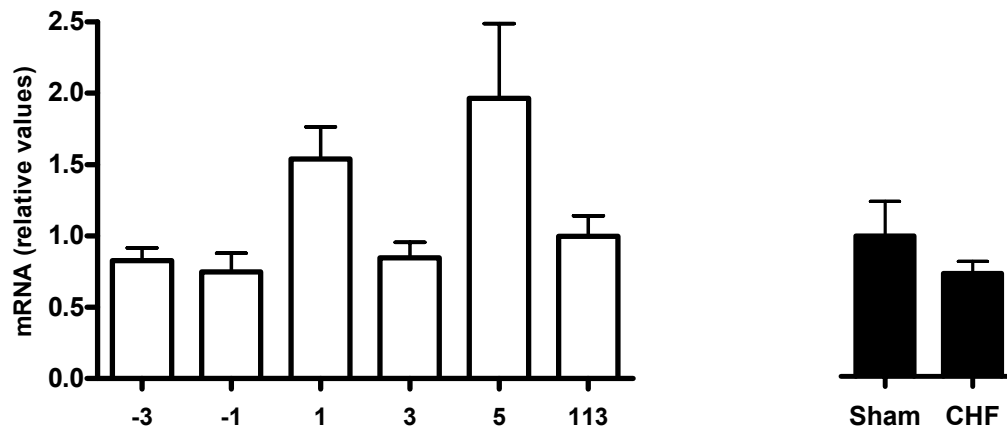


Figure 16: mRNA expression of 5-HTT in foetal, neonatal, adult and failing rat hearts quantified by the use of real-time quantitative RT-PCR. The foetal and neonatal groups (-3 days to 5 days) are compared to the 113-day-old rat hearts (left panel). Heart failure rats are compared to Sham (right panel). The values obtained are normalised to six normalisation genes (Table 3) by using the method of Vandesompele (2002). The groups of 113-day-old rats and Sham were assigned the value of 1.

4.3 Cardiomyocytes and non-cardiomyocytes from aorta-banded rats

To compare gene expression patterns in cardiac non-myocytes (non-cardiomyocytes, NCM) and myocytes (cardiomyocytes, CM) of failing rat hearts, mRNA expression of five different receptors (5-HT_{2A}, 5-HT_{2B}, 5-HT_{4(b)}, β_1 -AR and β_2 -AR) and ANP was quantified by the use of real-time quantitative RT-PCR in the two cell fractions from aorta-banded rat hearts.

Sham, hypertrophied (AB 1-3) and heart failure rats (ABHF) were included in the study. All the values obtained were normalised to GAPDH expression and Sham was assigned the value of 1 to simplify the comparison of groups.

4.3.1 The expression of ANP mRNA

The ANP mRNA expression level was quantified to confirm the pathological phenotype of the heart groups analysed.

Both in the NCM and CM of CHF rats, the ANP expression was highly increased. In the NCM the expression was elevated about 30-fold, and in the CM about 50-fold. There was also a significant increase in the hypertrophied rats for both cell types.

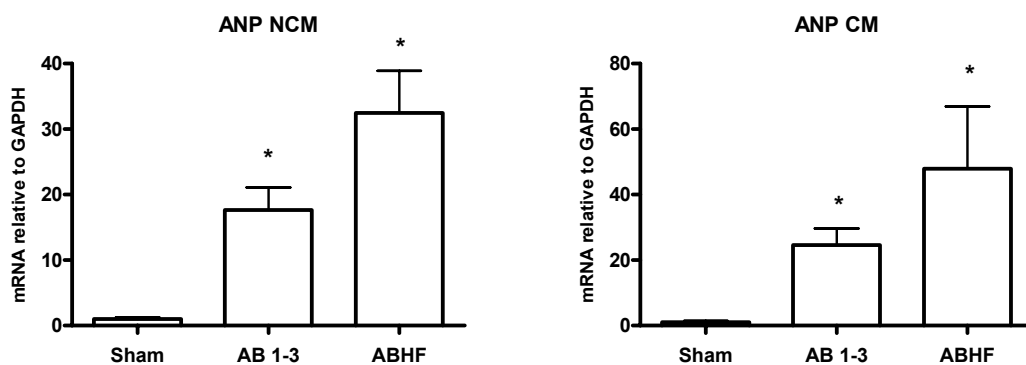


Figure 17: Expression of ANP mRNA in non-cardiomyocytes and cardiomyocytes of Sham, AB 1-3, and ABHF rats. mRNA was quantified by real-time quantitative RT-PCR and normalised to GAPDH. The left panel shows mRNA expression in NCM of Sham, AB 1-3 and ABHF rats, the right panel shows the expression in the CM. Sham was assigned the value of 1 and n=12 for all groups. * p < 0.05

4.3.2 Expression of the 5-HT_{4(b)} receptor mRNA

For 5-HT_{4(b)} there was an increase from the Sham through the AB 1-3 group and to the failing heart. The level was elevated 6-fold in both the NCM and CM of ABHF rats.

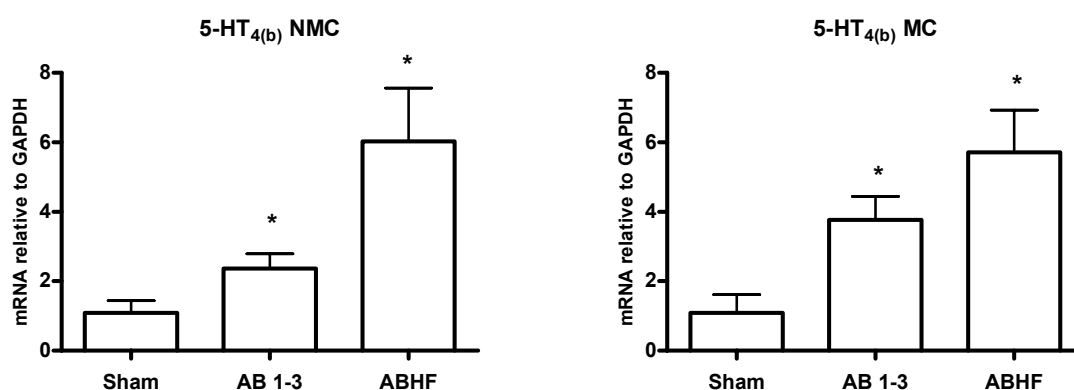


Figure 18: Expression of 5-HT_{4(b)} mRNA in non-cardiomyocytes and cardiomyocytes of Sham, AB 1-3 and ABHF rats. mRNA was quantified by real-time quantitative RT-PCR and normalised to GAPDH. The left panel shows mRNA expression in NCM of Sham, AB 1-3 and ABHF rats, the right panel shows the expression in the CM. Sham was assigned the value of 1 and n=12 for all groups. * p < 0.05 vs. Sham

4.3.3 Expression of the 5-HT_{2A} receptor mRNA

The mRNA expression of the 5-HT_{2A} receptor was increased in hypertrophied and heart failure rats, both in NCM and CM. In the NCM the amount of mRNA was unsignificantly doubled, compared to Sham, both in the AB 1-3 and ABHF group. For the CM the expression was significantly elevated 3-fold both in the AB 1-3 and ABHF group compared to Sham.

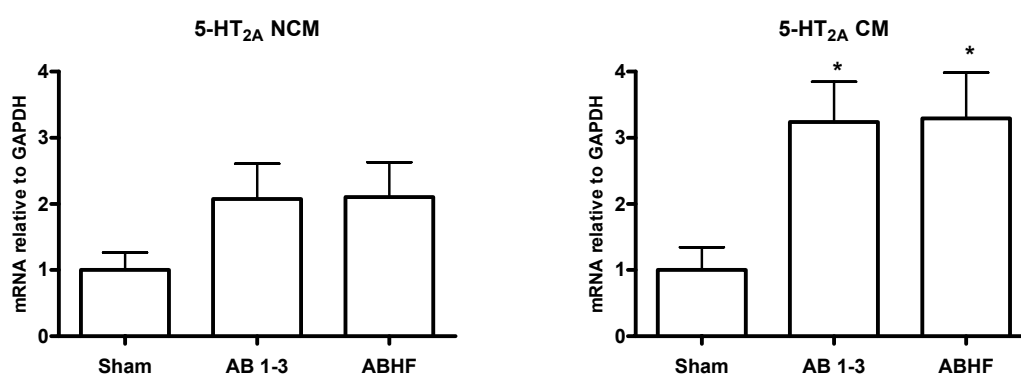


Figure 19: Expression of 5-HT_{2A} mRNA in non-cardiomyocytes and cardiomyocytes of Sham, AB 1-3 and ABHF rats. mRNA was quantified by real-time quantitative RT-PCR and normalised to GAPDH. The left panel shows mRNA expression in NCM of Sham, AB 1-3 and ABHF rats, the right panel shows the expression in the CM. Sham was assigned the value of 1 and n=12 for all groups. * p < 0.05 vs. Sham

4.3.4 Expression of the 5-HT_{2B} receptor mRNA

In the CHF group of the NCM, expression of the 5-HT_{2B} receptor was significantly elevated 4-fold, compared to both Sham and the AB 1-3 group. Concerning the CM, the level decreased by 50% going from Sham to AB 1-3 and ABHF rats, but this was not a significant decrease.

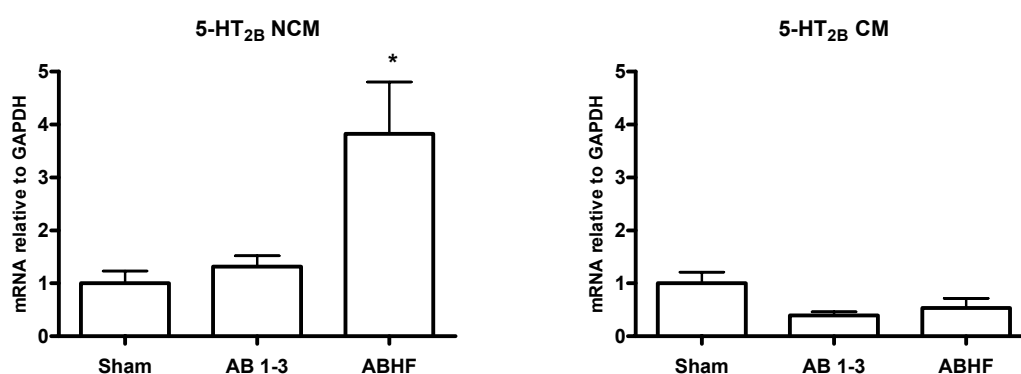


Figure 20: Expression of 5-HT_{2B} mRNA in non-cardiomyocytes and cardiomyocytes of Sham, AB 1-3 and ABHF rats. mRNA was quantified by real-time quantitative RT-PCR and normalised to GAPDH. The left panel shows mRNA expression in NCM of Sham, AB 1-3 and ABHF rats, the right panel shows the expression in the CM. Sham was assigned the value of 1 and n=12 for all groups. * p < 0.05 vs. Sham

4.3.5 Expression of the β_1 -AR mRNA

Among the β -adrenergic receptors the β_1 -AR is the most abundant one in mammalian heart and comprises about 70-80% of the total β -AR number. Both β_1 - and β_2 -ARs mediate positive inotropic response in heart, both in human and rat.

The β_1 -AR mRNA expression was decreased in the hypertrophied hearts and the failing ones, both in the NCM and the CM, however, not significantly.

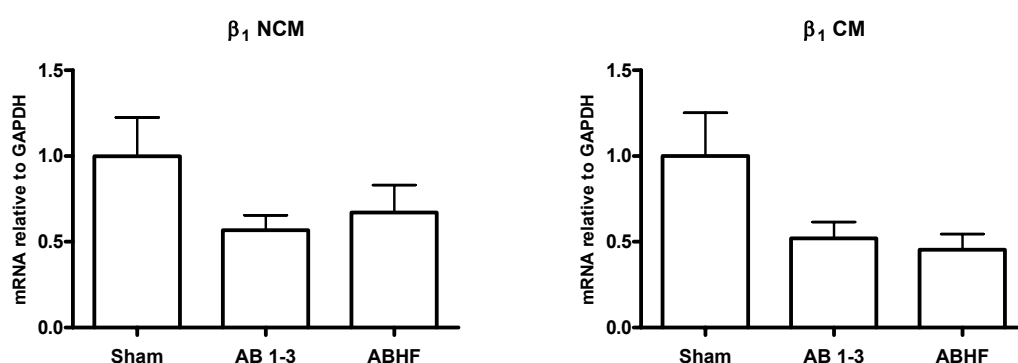


Figure 21: Expression of β_1 -AR mRNA in non-cardiomyocytes and cardiomyocytes of Sham, AB 1-3 and ABHF rats. mRNA was quantified by real-time quantitative RT-PCR and normalised to GAPDH. The left panel shows mRNA expression in NCM of Sham, AB 1-3 and ABHF rats, the right panel shows the expression in the CM. Sham was assigned the value of 1 and n=12 for all groups.

4.3.6 Expression of the β_2 -AR mRNA

The β_2 -ARs mediate positive inotropic response in heart. The receptors are primarily expressed in cells other than cardiac myocytes (e.g. endothelial cells, fibroblasts and vascular smooth muscle cells). However, they can mediate functional responses in cardiomyocytes. The receptors are responsible for causing smooth muscle relaxation in many organs, e.g. the bronchi.

For the β_2 -AR a significant increase in mRNA expression was detected in the NCM of the ABHF group. The expression was twice as high in this group, compared to Sham and hypertrophied hearts. In the CM the mRNA expression was equal in all three groups (Sham, AB 1-3 and ABHF).

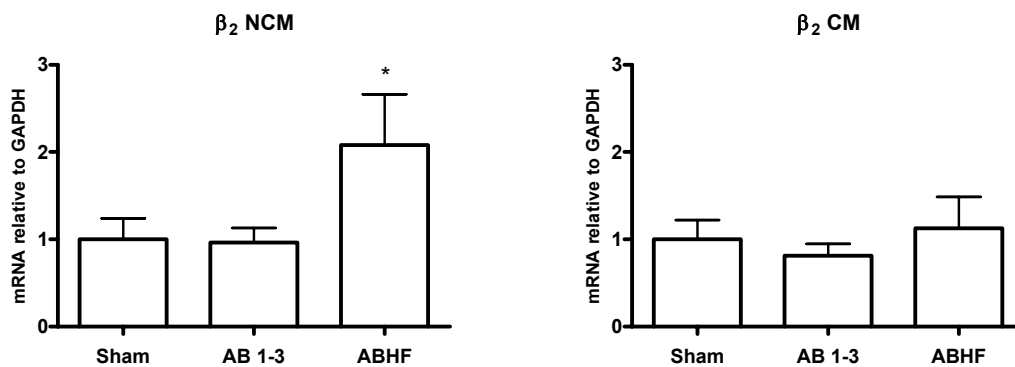


Figure 22: Expression of β_2 -AR mRNA in non-cardiomyocytes and cardiomyocytes of Sham, AB 1-3 and ABHF rats. mRNA was quantified by real-time quantitative RT-PCR and normalised to GAPDH. The left panel shows mRNA expression in NCM of Sham, AB 1-3 and ABHF rats, the right panel shows the expression in the CM. Sham was assigned the value of 1 and n=12 for all groups. * $p < 0.05$ vs. Sham

5. DISCUSSION

Before developing drugs it is necessary to fully understand the pathophysiology of heart failure as well as the underlying mechanisms. Increased interest is turned to find the genetic factors involved in heart failure. A hallmark in CHF is changes in the myocardial phenotype due to a reintroduction of a foetal gene program, with a pattern of gene expression different from that in adult myocardium. The profile of gene expression in the foetal and failing heart, however, is largely unknown.

5.1 Experimental considerations

In this study real-time quantitative RT-PCR was used to determine the mRNA level of different genes concerning the serotonergic system in left ventricle from foetal, neonatal and congestive heart failure rats. mRNA from cardiomyocytes and non-cardiomyocytes of heart failure rats were also included in the study. All values were normalised and compared to Sham operated and/or adult rats.

Real-time quantitative RT-PCR is no longer limited to specialist core facilities. It has become an established and common technique for quantifying mRNA in biological samples. Benefits of this procedure over conventional methods for measuring RNA include its sensitivity, large dynamic range, and the potential for high throughput as well as accurate quantification (Huggett et al., 2005).

The method is based on the assumption that there is a quantitative relationship between amount of starting target sequence and amount of PCR product at any given cycle. Since the quantitation relies on C_t values determined during the detectable exponential phase of the polymerase chain reaction, the effect of limiting reagents will not affect the results and the quantification is therefore more precise and reproducible than in regular PCR (Bustin, 2000). The variability of the data obtained from real-time quantitative RT-PCR reactions is also significantly lower than for regular PCR (Bustin, 2000).

Although real-time quantitative RT-PCR is widely used to quantify biologically relevant changes in mRNA levels, there remain a number of problems associated with its use. To

achieve quantitative benefit from the method, it is important to make use of the correct normalisation strategy. This is to control for experimental error introduced during the multistage process required to extract and process the RNA (Huggett et al., 2005). Several strategies have been proposed for normalising real-time RT-PCR data. These range from ensuring that similar samples sizes are chosen to using an internal housekeeping or reference gene. By using mRNA of reference genes you have a strategy that is simple to use, and you also have an internal control that is subjected to the same conditions as the mRNA of interest, also measured by real-time quantitative RT-PCR (Huggett et al., 2005).

GAPDH has been used as a normalisation gene in wide-ranging studies and its expression is claimed to be stable over time and not to be affected by experimental factors (Winer et al., 1999). However, GAPDH may not be as stable as first anticipated and its expression level seems to vary between different individuals, during the cell cycle and with developmental stage (Bustin, 2000). The latter point is of significant importance in this study analysing developing foetal and neonatal hearts in addition to failing ones; a pathophysiological condition associated with altered gene expression.

In the analysis of the foetal genes an Excel-based program was used to allow normalisation against multiple reference genes. geNorm allows selection of the most stable reference genes by use of geometric means of the expression of the candidate cDNA (Vandesompele et al., 2002). This is a robust method for providing accurate normalisation and is consequently favourable if fine measurements are to be made. However, it is not always possible to measure multiple reference genes due to limited sample availability and cost (Huggett et al., 2005).

According to Brattelid et al. (2005b) the chosen normalisation genes, additionally to GAPDH, used in the foetal gene program study are fairly stable ones and allow accurate estimation of the gene regulation.

Unlike DNA, which is as tough as old boots, RNA is extremely delicate once removed from its cellular environment. Its purification is much trickier than of DNA, and a template suitable for inclusion in an RT-PCR assay must be of highest quality if quantitative results are to be relevant (Bustin and Nolan, 2004). The assessment of RNA integrity by inspection of the 28S and 18S rRNA bands was accomplished by the use of both ordinary agarose gel

electrophoresis before DNase treatment, and capillary gel electrophoresis by the Agilent 2100 Bioanalyzer after DNase treatment.

Sample purity, and concentration as well, were also assessed spectrophotometrically by the Ultrospec 2100 Pro Spectrophotometer before DNase-treatment and by NanoDrop ND-1000 Spectrophotometer after DNase-treatment. Samples included in the study were mainly selected by the quality assessment by the NanoDrop and Bioanalyzer.

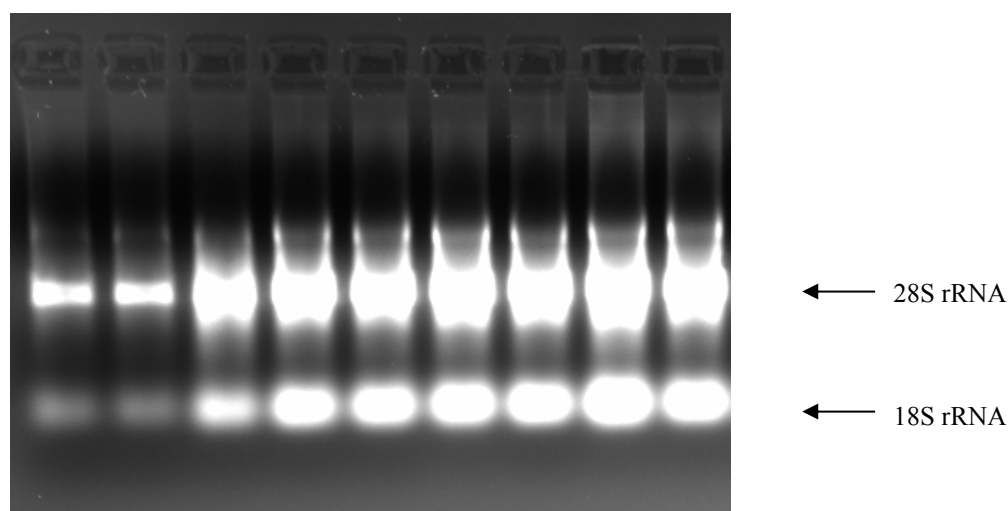


Figure 23: Agarose gel electrophoresis of a representative selection of samples.

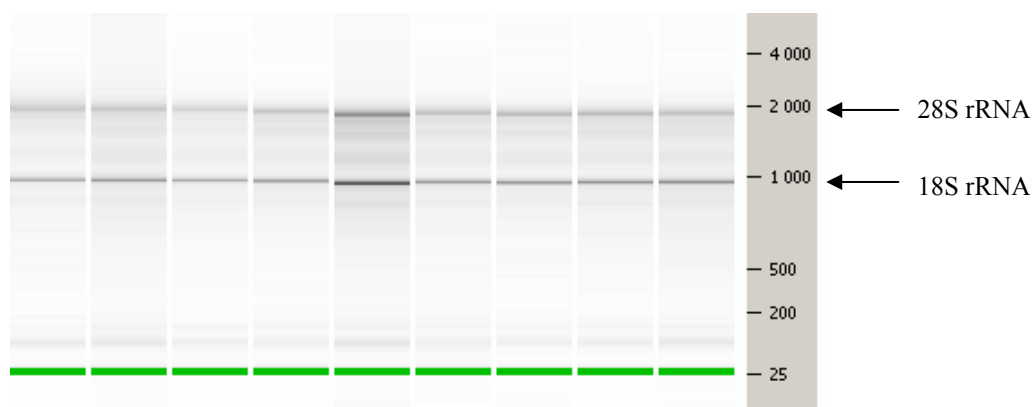


Figure 24: Representative example of Bioanalyzer results. The line in front (green) represents the marker. The next bands represent the 18S rRNA subunit and the 28S rRNA subunit, respectively.

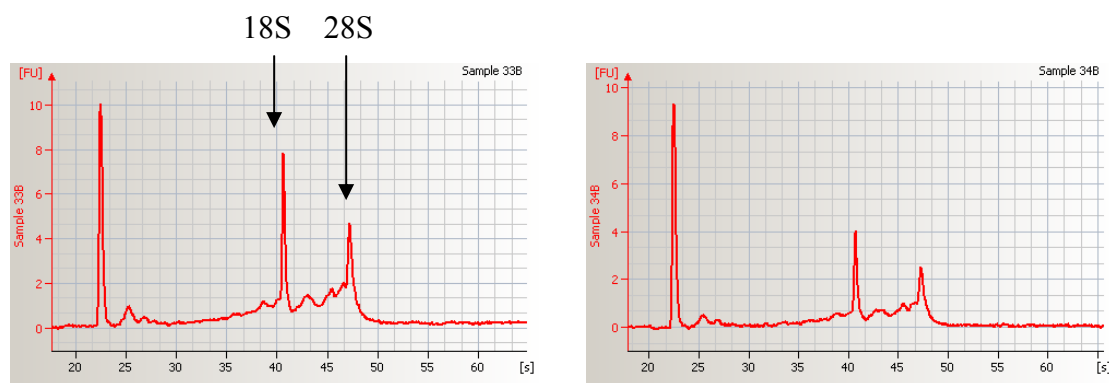


Figure 25: Examples of Bioanalyzer results showing electropherograms (chromatograms) of two representative samples included further in the study. The peak in front represents the marker. The next two peaks represent the 18S rRNA subunit and the 28S rRNA subunit, respectively.

5.2 The 5-HT_{4(b)} receptor

This study demonstrates for the first time expression of the 5-HT_{4(b)} receptor in the foetal and neonatal heart at levels significantly above what is observed in adult heart. The relatively high 5-HT_{4(b)} receptor expression in foetal heart, declining with development to a minimum in adult heart, suggests a role of this receptor in cardiac development. The role of the receptor in the developing heart remains unknown, but preliminary data in our lab indicates that the foetal and neonatal 5-HT_{4(b)} receptor mediates a positive inotropic effect. In the failing heart the 5-HT_{4(b)} receptor mRNA expression was increased to levels comparable to foetal and neonatal expression. An increase in 5-HT_{4(b)} receptor mRNA in CHF is in line with previous data from our group, in two different animal models of heart failure. Both myocardial infarction and aortic banding-induced heart failure models show increased 5-HT_{4(b)} receptor mRNA levels in failing heart (Qvigstad et al., 2005a) (Brattelid et al., 2005a). A hallmark of heart failure is the transition in gene expression towards a foetal genotype correlated with loss of cardiac functions (Francis and Tang, 2003). Although the expression is not as high as in the foetal hearts, the level is comparable to the neonatal expression level. This suggests that the 5-HT_{4(b)} receptor may be part of a foetal gene program reactivated in heart failure.

Upregulation of 5-HT_{4(b)} mRNA levels occurred in both CM and NCM fractions similar to the changes observed in LV tissue (Brattelid et al., 2005a). The CM localised increase in 5-HT_{4(b)} expression can explain the increase in 5-HT_{4(b)}-mediated inotropic response to serotonin in the failing heart (Brattelid et al., 2004b) (Brattelid et al., 2005a) (Qvigstad et al., 2005a). Due to

the low level of 5-HT_{4(b)} receptors in heart, and ventricle in particular, it is difficult to determine the 5-HT_{4(b)} receptor level in ventricle by use of radioligands as well as antibodies. The increase in CM 5-HT_{4(b)} expression level advocates that the 5-HT inotropic response in failing hearts is mediated by the 5-HT_{4(b)} receptor. The possible role of 5-HT_{4(b)} receptors in NCM is unknown. The presence of up to 14 % of CM in the NCM fraction might have introduced an artificial level of the 5-HT₄ gene expression as observed in the NMC fraction. Suggesting that the 5-HT₄ is expressed in the CM fraction only, entails that the 5-HT₄ expression level is underestimated in the CM fraction, due to the apparently high level of NCMs in the CM fraction. Although speculative, polynucleation of cardiomyocytes might increase the “genetic power” of the cardiomyocytes compared to the non-cardiomyocytes.

5.3 The 5-HT_{2A} receptor

The almost identical 5-HT_{2A} mRNA expression level in foetal, adult, Sham operated and CHF rats suggests a minor role of this receptor in chronic heart failure. However, the significant increase in 5-HT_{2A} observed at the time of birth might imply an important role of 5-HT_{2A} receptor in acute heart failure.

In acute CHF (dominated by the onset of hypertrophy) the 5-HT_{2A} mRNA expression and function is significantly increased (Qvigstad et al., 2005b). Likewise, the 5-HT_{2A} receptor is increased significantly at both the functional and mRNA level in hypertrophic hearts in a rat aortic banding model (Brattelid et al., 2005a). Both acute CHF and aortic banding is associated with an increase in wall stress. The wall stress is also increased at the time of birth as a result of significant rearrangement of the cardiac circulation due to onset of lung respiration and closure of *foramen ovale*. For this reason neonatal hearts of newborns are also exposed to an acute increase in wall stress associated with cardiac growth during the development of the heart. The increased 5-HT_{2A} receptor mRNA expression level is therefore possibly induced by the acute increase in wall stress at the time of birth. The acute increase in 5-HT_{2A} receptor mRNA does not seem to last, and the 5-HT_{2A} expression level is gradually decreased again back to a foetal and adult level in the early neonatal development.

In hypertrophic and failing hearts of aorta-banded rats both the CM and NCM fractions displayed a significant increase in 5-HT_{2A} receptor mRNA level. The aorta-banded rats are exposed to an increased left ventricular wall stress. This is in line with earlier findings indicating a role of the 5-HT_{2A} receptor in acute and longstanding hypertrophic heart failure (Qvigstad et al., 2005b) (Brattelid et al., 2005a).

The increase in mRNA level of the 5-HT_{2A} receptor at birth and in the hypertrophic heart could reflect a role of this receptor in the response to the increased wall stress.

5.4 The 5-HT_{2B} receptor

The coincidence of increased 5-HT_{2B} mRNA and increased wall stress at birth supports previous studies in transgenic (Nebigil et al., 2003) and knockout (Nebigil et al., 2000) (Nebigil et al., 2001) mouse models. Nebigil et al. (2000) provided genetic evidence that 5-HT via 5-HT_{2B} receptor regulates differentiation and proliferation of developing and adult heart. Knocking out the 5-HT_{2B} gene leads to embryonic and neonatal death caused by heart defects. Nebigil et al. (2001) further showed that ablation of the 5-HT_{2B} receptor leads to dilated cardiomyopathy and provides strong genetic evidence that serotonin via the 5-HT_{2B} receptor regulates cardiac structure and function. On the other hand, overexpression of 5-HT_{2B} receptor in heart leads to compensated hypertrophic cardiomyopathy accompanied by abnormal mitochondrial proliferation and enzyme activity. These mice have hypertrophy as a result of increased number of cardiomyocytes and increased growth (Nebigil et al., 2003). The acute increase in wall stress at birth and increased 5-HT_{2B} receptor mRNA level is consistent with a role of the 5-HT_{2B} receptor in cardiac hypertrophy. However, in the hypertrophic hearts analysed, the 5-HT_{2B} receptor increased 4-fold in the failing NCM fraction, suggesting a role of this receptor mainly in the non-contractile tissue. It has recently been demonstrated that the 5-HT_{2B} receptor induces hypertrophy by control of cytokine production in ventricular fibroblasts (Jaffre et al., 2004). This suggests a role of the 5-HT_{2B} receptor in hypertrophy-induced heart failure, consistent with induction of mRNA expression of this receptor primarily in fibroblasts.

5.5 The serotonin transporter

The serotonin transporter expression level is regulated neither throughout foetal and neonatal development, nor in heart failure. SERT was analysed because it is the key mechanism controlling serotonin concentration in major heart diseases. A regulation of the SERT in cardiac tissue could have been an indication of local regulation of the 5-HT level with functional implications on the 5-HT₄-mediated inotropic effect. The importance of SERT in heart development has been demonstrated in SERT knock out mice where the left ventricle decreased compared to normal mice (Eddahibi et al., 2000). The reduced heart size of the SERT knock out mice did not affect the hemodynamic parameters analysed and is therefore probably not a master key in heart development. Although the SERT is not regulated in the developing heart and heart failure, Sari and Zhou (2003) demonstrated that SERT is important for regulation of the 5-HT level in the development of foetal heart cells and its implication in the cell proliferation.

5.6 The β -adrenergic receptors

The β_1 -adrenergic receptor mRNA expression was not regulated in either the hypertrophied or the failing heart NCM and CM fractions, whereas the β_2 -adrenergic receptor mRNA expression increased in the failing NCM fraction only. In human heart failure there is a loss of responsiveness (desensitisation) to β -AR stimulation by catecholamines associated with a decrease in the receptor density (Brodde and Michel, 1999). The lack of a significant down regulation of β_1 -AR mRNA in heart failure is at variance with some previous reports (Böhm et al., 1990) (Brodde and Michel, 1999) (Brodde et al., 2001) but supported at the receptor level by Sjaastad et al. (2003) who did not find any differences of total β -AR binding between CHF and Sham in a myocardial infarction rat model. Gu et al. (1998) demonstrated regional differences in the β -AR expression in the failing heart at the protein level with a significant down regulation of the receptors in the infarction area and the border zone and no change in the viable area of the failing heart compared to Sham. The gene expression of the failing heart might therefore be affected by the etiology leading to heart failure. A direct comparison of the results obtained here in the aorta-banded heart failure model might differ with the myocardial infarction induced heart failure.

The 2-fold increase in β_2 -AR mRNA expression in NCM of ABHF is difficult to explain. The anticipated inotropic role of β_2 -AR in heart suggests that expression of the receptor should at least occur in the CM. However, the β_2 -AR mRNA expression levels were not changed in hypertrophic and failing heart, and the increase of this receptor in NCM can therefore not be explained by contamination of CM in the NCM fraction. The NCM β_2 -AR expression needs further investigations.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

- ♥ The high 5-HT_{4(b)} mRNA expression in CHF, foetal and neonatal cardiac ventricle is consistent with 5-HT_{4(b)} receptor as a representative of the foetal gene program reactivated in CHF.
- ♥ The foetal expression of 5-HT_{4(b)} receptor mRNA is consistent with a possible role for the 5-HT₄ receptor in cardiac development.
- ♥ The increase in 5-HT_{2A} receptor mRNA is associated with birth, consistent with a possible role of 5-HT_{2A} receptor in the hypertrophic response.
- ♥ The transitory increase in the 5-HT_{2B} receptor mRNA at birth might be involved in the hypertrophic response of the acutely stretched heart.
- ♥ The serotonin transporter is not regulated throughout foetal and neonatal development or in heart failure.
- ♥ 5-HT_{2A} and 5-HT_{4(b)} receptors are induced in cardiomyocytes in myocardial hypertrophy and CHF after banding of the ascending aorta.
- ♥ Increased 5-HT_{2B} mRNA expression in hypertrophic and failing ventricles occurs primarily in the non-cardiomyocyte cell population.

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8. APPENDIX

8.1 Materials and recipes

8.1.1 Chemicals and reagents

| Chemical/Reagent | Abbreviation | Manufacturer | Cat. No. |
|---|--------------|----------------------|----------------|
| 2X Master Mix | | Eurogentec | RT-QP2X-03-50+ |
| Absolute (100%) ethanol | | Arcus | - |
| Ammonium acetate, 5M | | Ambion | 9071 |
| Chloroform | | SDS | C-2432 |
| dNTP Mix, 10mM** | | Invitrogen | 18427-013 |
| Ethanol (96%) | | Arcus | - |
| Ethidium bromide | EtBr | Sigma | E8751 |
| Formamide | | Sigma | F7508 |
| Glycoblue | | Ambion | 9515 |
| Isopropanol | | Arcus | 1-9516 |
| Loading buffer** | | - | - |
| Oligo (dT) ₁₂₋₁₈ | | Invitrogen | - |
| Primers | | Invitrogen | - |
| Primers and Probe mixes | | Applied Biosystems | - |
| Probes | | Eurogentec | - |
| RNA 6000 Ladder | | Ambion | 7152 |
| RNAlater | | Ambion | 7021 |
| Nuclease-free water (not DEPC treated) | | Ambion | 9932 |
| RNA 6000 Nano LabChip kit* | | Agilent Technologies | 5065-4476 |
| RNaseOut Recombinant Ribonuclease Inhibitor, 40U/μl | | Invitrogen | 10777-019 |
| RNaseZAP | | Ambion | 9780 |
| RQ1 RNase-free DNase (1U/μl)* | | Promega | E3730 |
| SeaKem LE Agarose | | Cambrex | 50004 |
| SuperScript III RNase H-Reverse Transcriptase, 200U/μl* | | Invitrogen | 18080-044 |
| TAE buffer** | | - | - |
| Trizol | | Invitrogen | 15596-018 |

*Content of commercial kits is specified in 8.1.2 **Content of reagents is specified in 8.1.3

8.1.2 Content of commercial kits

- **RQ1 RNase-free DNase**

- RQ1 RNase-free DNase 10X Reaction Buffer (400mM Tris-HCl (pH 8.0 at 25°C), 100mM MgSO₄, 10 mM CaCl₂)
- RQ1 RNase-free DNase (1U/μl)
- RQ1 DNase Stop Solution (20mM EGT at 25°C)

- **RNA 6000 Nano LabChip kit**

RNA 6000 Nano Chips

- Nano Chips
- Electrode Cleaners

RNA 6000 Nano Reagents & Supplies (reorder number 5065-4475, Agilent Technologies)

- RNA Nano Dye Concentrate
- RNA 6000 Nano Marker
- RNA 6000 Nano Gel Matrix
- Spin filters

Tubes for Gel-Dye Mix

- Safe-Lock Eppendorf Tubes PCR Clean (DNase-/RNase- free)

- **SuperScript III RNase H-Reverse Transcriptase**

- SuperScript III RT, 200 U/μl
- 5X First-Strand Buffer (250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM MgCl₂)
- 0.1 M DTT

8.1.3 Content of reagents

| Reagent | Ingredients |
|----------------|--|
| dNTP Mix, 10mM | Same amount dATP, dGTP, dCTP and dTTP |
| Loading buffer | 1 mM EDTA 0.25% bromophenol blue 0.25% xylene cyanol FF 50% glycerol Stored at 4°C |
| TAE buffer | 0.04 M Tris-acetate 1 mM EDTA |

8.2 Protocols

8.2.1 Homogenisation, phase separation, RNA precipitation and washing

1. Wash 100 mg of tissue in NaCl and tear it apart into small pieces. Homogenise in 1.5ml of Trizol together with a ceramic particle by using a shaker machine (Retsch MM301). Use 2ml tubes.
2. Remove insoluble material from the homogenates by centrifugation at 12000 x g for 10 minutes at 2-8°C by the use of Biofuge *fresco* (Heraeus).
3. Incubate the homogenates for 5 minutes at 15-30°C (room temperature).
4. Spin the Phase Lock Gel tubes for about 30 seconds to collect the Phase Lock Gel to pellet.
5. Transfer the clear supernatant from the homogenates to the Phase Lock Gel tubes.
6. Add 200 µl of chloroform per 1 ml of Trizol and shake vigorously by hand for 15 seconds.
7. Incubate for 2-3 minutes at 15-30°C.
8. Centrifuge the samples at 12000 x g for 15 minutes at 2-8°C.
9. Transfer the colourless upper aqueous phase to fresh tubes.
10. Precipitate the RNA by mixing with 500µl isopropanol per 1ml of Trizol.
11. Incubate samples at 15-30°C for 10 minutes.
12. Centrifuge at 12000 x g for 10 minutes at 2-8°C.
13. Remove the supernatant and wash the RNA pellet once with 1ml 75% ethanol per 1ml of Trizol.
14. Mix the sample by vortexing and centrifuge at 7500 x g for 5 minutes at 2-8°C.
15. Remove the ethanol and let the pellets air-dry for 5-10 minutes.
16. Dissolve RNA in RNase-free water by passing the solution a few times through a pipette tip.
17. Incubate on a heating block (BLOCKHEATER, Stuart Scientific) for 10 minutes at 55-60°C to dissolve the RNA completely.
18. Pipette aliquots for spectrophotometric determination and agarose gel electrophoresis.
19. Add 1µl of RNaseOut per 20µl sample volume and mix by using the pipette.

8.2.2 Spectrophotometric determination of RNA purity and concentration by the use of Ultrospec 2100 pro

1. Mix 5µl RNA solution (before adding RNaseOut in step 19 in protocol description 8.2.1!) with 95µl RNase-free water.
2. Calibrate the UV-detector by using 100µl of RNase-free water.
3. Pipette 100µl diluted sample into the cuvette.
4. Make the measurements.

8.2.3 Agarose gel electrophoresis

1. Mix 5µl of the sample (before adding the RNaseOut in step 18 in protocol description 8.2.1!) with 2µl formamide.
2. Mix 0.8g agarose (SeaKem LE Agarose, Cambrex) with 50ml 1X TAE buffer (or 1.3g agarose in 100ml buffer for a bigger gel run) and bring it to the boiling point several times until dissolved by the use of a microwave oven. Mix thoroughly after each parboiling.
3. Add 7µl of ethidium bromide (or 14µl for a bigger gel). Mix thoroughly.
4. Cast the gel in a horizontal tray.
5. Heat the samples at 70°C for 5-10 minutes.
6. Cool on wet ice for a couple of minutes.
7. Add 2µl of loading buffer to each sample and spin down.
8. Load the samples on to the gel.
9. Use a voltage of 50V and let the samples run for about 45 minutes. Make sure that the poles are right.
10. Picture the gel by the use of UV light (Epi Chemi II Darkroom, UVP).

8.2.4 DNase treatment of RNA

1. Set up the DNase-reaction as follows:

| | |
|---|------|
| RNA in RNase-free water | 25µg |
| RQ1 RNase-free DNase 10X Reaction Buffer (See note 1) | 5µl |
| RQ1 RNase-free DNase (1U/µl) (See note 2) | 5µl |
| RNase-free water to a final volume of | 50µl |

Notes:

- 1) 1µl reaction buffer per 10µl total sample volume.

- 2) 1µl DNase (1U/µl) per 5µg DNA.
2. Incubate at 37°C for 30 minutes by the use of a heater (Termaks, KEBOLab).
3. Add 1µl of DNase Stop Solution per 10µl total sample volume to terminate the reaction.
4. Incubate at 65°C for 10 minutes to inactivate the DNase.

8.2.5 Precipitation of RNA after DNase treatment

1. Add the following components to the DNase treated RNA samples:
 - 0.1 volume 5M ammonium acetate
 - 1µl Glycoblue
 - 2.5 volumes absolute (100%) ethanol
2. Mix thoroughly by vortexing.
3. Incubate at -20°C over night or at -70°C for 30 minutes.
4. Recover the RNA by centrifugation at 12000 x g for 30 minutes at 4°C.
5. Remove the supernatant carefully using a pipette.
6. Centrifuge the tubes briefly one more time and remove the remaining fluid.
7. Add 1ml of 75% ethanol to wash the pellets. Shake carefully.
8. Centrifuge at 12000 x g for 10 minutes at 4°C.
9. Remove the supernatant as in steps 5 and 6.
10. Dry the pellets for a few minutes.
11. Add 20µl of RNase-free water to dissolve the RNA.
12. Pipette aliquots for spectrophotometric determination by the use of NanoDrop.
13. Add 1µl RNaseOut per 20µl sample volume to the remaining RNA solutions. Mix by using the pipette tip.
14. Freeze at -70°C.

8.2.6 Determination of RNA purity and concentration by the use of NanoDrop ND-1000 Spectrophotometer

1. Mix 2µl RNA solution (before adding the RNaseOut!) with 8µl RNase-free water.
2. Run a blank by the use of 2µl RNase-free water.
3. Use volumes of 2µl and run each sample twice.

8.2.7 The Agilent 2100 Bioanalyzer; Determination of RNA quality

Allow all reagents to equilibrate to room temperature for 30 minutes before use! Wear gloves.

Decontaminating the electrodes

1. Slowly fill one of the wells of an electrode cleaner with 350µl RNaseZAP (Ambion).
2. Place the electrode cleaner in the Bioanalyzer, close the lid and leave it there for about 1 minute.
3. Slowly fill one of the wells of another electrode cleaner with 350µl RNase-free water.
4. Place the electrode cleaner in the Bioanalyzer and leave it there for about 10 seconds. Open the lid, remove the electrode cleaner and wait another 10 seconds before you close the lid again. (Save the electrode cleaner for cleaning after the run.)

Preparing the gel

1. Place 550µl of RNA 6000 Nano gel matrix (Agilent Technologies) (red) into the top receptacle of a spin filter and spin for 10 minutes at $1500 \times g \pm 20\%$ (corresponding 4000rpm).
2. Discard the filter and aliquot 65µl filtered gel into 0.5ml microcentrifuge tubes (Safe-Lock Eppendorf Tubes PCR Clean (DNase-/RNase-free), Agilent Technologies). Use the aliquots within one month of preparation.

Preparing the gel-dye mix

1. Vortex RNA 6000 Nano dye concentrate (Agilent Technologies) (blue) for 10 seconds and spin down.
2. Add 1µl of dye to a 65µl aliquot of filtered gel.
3. Vortex and visually inspect proper mixing of gel and dye.
4. Spin tube for 10 minutes at room temperature at $13000 \times g$ (14000 rpm). Use prepared gel-dye mix within one day.

Loading the gel-dye mix

1. Take a new RNA Nano Chip (Agilent Technologies) out of its sealed bag and place it on the Chip Priming Station (Agilent Technologies).

2. Pipette 9.0µl of the gel-dye mix into the well marked G. Insert the tip of the pipette to the bottom of the chip well when dispensing!

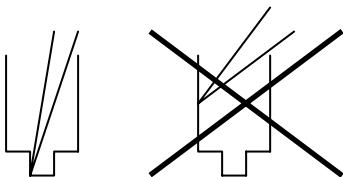


Figure 26: How to hold the pipette tip. It is very important to hold the pipette tip as shown to the left to avoid bubbles in the wells.

3. Set the timer to 30 seconds. Make sure that the plunger is at 1ml and then close the Chip Priming Station.
4. Press the plunger until it is held by the syringe clip and wait for exactly 30 seconds and then release the plunger with the clip release mechanism. Wait for 5 seconds and then slowly pull back the plunger to the 1ml position. Open the Chip Priming Station.
5. Pipette 9.0µl of the gel-dye mix in each of the wells marked **G**. Discard the remaining gel-dye mix.

Loading the RNA Nano Marker

1. Pipette 5µl of the RNA 6000 Nano Marker (green) into the well marked with ladder symbol and each of the 12 sample wells. Do not leave any wells empty! Add 6µl (instead of 5µl) of the RNA 6000 Nano Marker to each unused sample well.

Loading the ladder and samples

1. Aliquot the amount of RNA 6000 ladder that you will use within a day into a RNase-free microcentrifuge tube and heat denature it for 2 minutes at 70°C before use.
2. Pipette 1µl of the ladder into the well marked with the ladder symbol.
3. To minimise the secondary structure, heat denature (70°C for 2 minutes) the samples before loading on the chip.
4. Pipette 1µl of each sample into each of the 12 sample wells.
5. Place the chip in the adapter of the vortex mixer. Vortex for 1 minute at the IKA Vortexer Mixer (Agilent Technologies; set-point 2400rpm).
6. Start the Agilent 2100 Bioanalyzer run within 5 minutes.

The run (www.agilent.com/chem/labonachip)

1. The samples move through the microchannels from the sample wells.
2. The samples are injected into the separation channel.
3. RNA fragments are separated according to their size by means of molecular sieving.
4. RNA fragments are detected by fluorescence at the detection point and results are recorded and analyzed with the Agilent 2100 Bioanalyzer software.
5. Once detected the samples pass into the waste/buffer well.

Cleaning up after the RNA Nano Chip Run

1. When the run is finished, remove the RNA Nano Chip from the Bioanalyzer immediately.
2. Place the electrode cleaner with RNase-free water in the Bioanalyzer and leave it there for about 10 seconds. Open the lid, remove the electrode cleaner and wait another 10 seconds before closing the lid again.

For further details see Agilent Technologies (2003).

8.2.8 First strand cDNA synthesis

1. Add the following components to nuclease-free microcentrifuge tubes:

| | | | |
|--|--------|---|-------|
| Oligo (dT) ₁₂₋₁₈ | 2 µl | } | Mix 1 |
| dNTP Mix, 10mM (same amount dATP, dGTP, dCTP and dTTP, Invitrogen) | 2 µl | | |
| Total RNA in RNase-free water | 7.5 µg | | |
| RNase- free water to a total volume of | 26 µl | | |
2. Heat at 65°C for 5 minutes using a heating block (PTC-100 Programmable Thermal Controller, MJ Research) and incubate on wet ice for a couple of minutes.
3. Collect the contents of the tubes by using a centrifuge (Varifuge 3.0R, Heraeus Instruments).
4. Add the following components:

| | | | |
|---|------|---|-------|
| 5X First-Strand Buffer | 8 µl | } | Mix 2 |
| 0.1 M DTT | 2 µl | | |
| RNaseOut Recombinant RNase Inhibitor (40U/µl) | 2 µl | | |
| SuperScript III RT (200 U/µl) | 2 µl | | |
5. Mix gently by pipetting.

6. Incubate at 25°C for 5 minutes, then at 50°C for 60 minutes. Finally inactivate the reaction by heating at 70°C for 15 minutes. Cool. All this can be done by using a PTC-100 Programmable Thermal Controller (MJ Research).

For all the samples parallel reactions should be run in the absence of reverse transcriptase. Replace the reverse transcriptase and the RNaseOut volume with RNase-free water.

8.2.9 Design of primers and probes in Primer Express

Guidelines for primer design

1. The melting point of the primers should be 58-60°C.
2. No more than two (three) Gs and/or Cs in the last five nucleotides at the 3' end of the primers are accepted.
3. Design primers as close to the probe as possible.

Guidelines for probe design

1. The melting temperature of the probe should be 68-70°C.
2. Select the strand that gives the probe more Cs than Gs. (The probe can be run both ways.)
3. The first base at the 5' end can not be a G. The G will then be able to function as a quencher.

8.2.10 Real-time quantitative RT-PCR

1. Dilute each sample of template (including the standards) with 60µl of RNase-free water.
2. Make a second dilution; Pipette 3µl of the template dilution already made and mix with 295µl of RNase-free water. Use this second dilution when looking at highly expressed genes.

Store the dilutions at -20°C.

3. Mix the following volumes (μl) of the given components:

| | |
|--|---|
| RNase-free water | $5.80 \times (3 \times \text{number of samples} + 2)$ |
| Primer 1 (forward) ($10\mu\text{M}$) | $0.66 \times (3 \times \text{number of samples} + 2)$ |
| Primer 2 (reverse) ($10\mu\text{M}$) | $0.66 \times (3 \times \text{number of samples} + 2)$ |
| Probe TaqMan ($5\mu\text{M}$) | $0.88 \times (3 \times \text{number of samples} + 2)$ |
| 2X Master Mix (Eurogentec) | $11.0 \times (3 \times \text{number of samples} + 2)$ |

4. Pipette $57\mu\text{l}$ of this reaction mix and add $9\mu\text{l}$ of template dilution. Use 0.2ml RNase-free microcentrifuge tubes (Sarstedt). This volume of $66\mu\text{l}$ is now a triplicate master. Keep on ice while working.
5. Use 384-well PCR plates and pipette $3 \times 20\mu\text{l}$ from each triplicate master.
6. Cover the PCR plate with optically clear ceiling tape (Sarstedt) and centrifuge by using a Varifuge 3.0R to collect the samples and remove air bubbles.
7. Run real-time quantitative PCR (ABI Prism 7900HT Sequence Detection System). Run 40 cycles, or 50 when looking at smaller amounts of mRNA. The run takes about 2 hours (or more when 50 cycles).